EXPRESSION AND FUNCTION OF THE FORMYL PEPTIDE RECEPTOR 2 IN EXPERIMENTAL MYOCARDIAL INFARCT

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ABSTRACT

In Acute Myocardial Infarction (AMI), inflammation is a prerequisite for healing but it can paradoxically extend tissue injury; hence it needs to be modulated. Here, we investigated the role of the pro resolving GPCR FPR2/ALX and its agonist Annexin A1 (AnxA1) in AMI using mice lacking of the Fpr2/3 genes and with an in-frame GFP gene 'knocked-in'.

We developed protocols aimed to determine GFP expression as an indication of Fpr2 gene activity. Also, the Left Anterior Descending Coronary Artery of male Fpr2/3 KO and littermate controls (WT) was occluded for 30min and re-opened for 90min. At the end tissue injury and inflammatory response were studied.

A significant proportion of Fpr2/3 KO perished during the procedure. The rest survived up to 90 min and exhibited a larger infarct size, with higher troponin I and inflammation markers (KC, TNFα) than WT animals. At the end of reperfusion, Fpr2/3 KO displayed an unbalanced production of pro and anti-inflammatory lipids (higher PGE_2, PGI_2, LTB_4 and attenuated PGA_1, RvD_2, LXA_4) and a deregulated activation of the cardioprotective IL-6/JAK/STAT3 signalling. Administration of AnxA1 afforded cardioprotection (reduction of infarct size; Troponin I, Caspase3 activity and TNFα) in WT but not in Fpr2/3 KO.

A parallel in vitro investigation on the functional FPR2/ALX domains required by AnxA1 and other agonists was also conducted. HEK-293 cells transfected with FPR1, FPR2/ALX and FPR1/FPR2 chimeric receptor were used and calcium flux,

pERK and gene modulation analysed. AnxA1 required the N-terminus and the II and III extracellular loops of FPR2/ALX to evoke canonical responses. SAA interacted/activated the I and the II extracellular loops of FPR2/ALX, whereas the compound 43 suffices the I extracellular loop.

In summary, the FPR2/AnxA1 pathway exerts a protective role in AMI. AnxA1 mimetic that activated selective FPR2/ALX domains can be synthetize to prevent tissue damage caused by AMI.

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Abbreviations

AA	Arachidonic acid
AAR	Area at risk
ACE	Angiotensin converting enzyme
AF2	Antiflammin 2
AhR	Aryl hydrocarbon receptor
AMI	Acute Myocardial Infarct
ANOVA	Analysis of variance
AnxA1	Annexin A1
APC	Antigen Presenting Cells
ATP	Adenosine triphosphate
Akt	Protein Kinase B
BCA	Bicinchoninic acid
CAMs	Cell adhesion molecules
CCR2	Chemokine receptor 2
CysLT1	Cysteinyl leukotriene receptor type 1
CT-1	Cardiotrophin 1
CHD	Coronary Heart Diseases
CytP450	Citocromo P450
COX	Cyclooxygenases
CNTF	Cilliary neurotrophic factor
C43	Compound 43
$cPLA_2$	Cytoplasmatic phospholipase A_2
DHA	Docosahexaenoic acid
DHGLA	Dihomo-Y-linoleic acid
ECM	Extracellular matrix
ECG	Electrocardiogram
EET	Epoxyeicosatetraenoic acids
FGF	Fibroblast growth factor receptor
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPA	Eicosapentaenoic acid
ERK	Extracellular-signal-regulated kinases

fMLF	N-formylmethionyl-leucyl-phenylalanine
FPRs	Formyl Peptide Receptors
FPR2	Formyl Peptide Receptor 2
FPR1	Formyl Peptide Receptor 1
GFP	Green fluorescent protein
GPCR	G protein coupled receptors
H&E	Haematoxylin and eosin
HEPE	Hydroperoxyyeicosatetraenoic acid
HEK	Human Embryonic Kidney
HDL	High density lipoprotein
IF	Infarct size
IL	Interleukin
IRI	Ischemia-reperfusion injury
JAM	Junctional Adhesion Molecule
JAK	Janus kinase
KO	Knockout
LADCA	Left anterior descending coronary artery
LIF	Leukaemia inhibitory factor
LT	Leukotriene
LO	Lipoxigenase
LOX	Lipoxigenase
LX	Lipoxins
LV	Left ventricle
LFA1	Lymphocyte function-associated antigen 1
MAC1	Macrophage receptor 1
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemoattractant protein-1
MFI	Median fluorescence intensity
MPO	Myeloperoxidase
NBT	p-nitroblue tetrazolium
OSM	Oncostatin M
PCR	Polymerase Chain Reaction
PECAM-1	Platelet endothelial cell adhesion molecule

PGI_2	Prostacyclin I_2
PG	Prostaglandin
PI3K	Phosphatidylinositol-3-kinase
PLA_2	Phospholipase A_2
PMN	Polymorphonuclear leukocytes
PPCI	Primary percutaneous coronary intervention
PSGL1	P-selectin glycoprotein ligand 1
PTCA	Percutaneous transluminal coronary angioplasty (PTCA
PUFAs	Polyunsaturated fatty acids
PKC	Protein kinase
$sPLA_2$	Secretory phospholipase A_2
RCTs	Randomized control trials
ROS	Reactive oxygen species
RvD_1	Resolvin D_1
RvD_2	Resolvin D_2
RvE_1	Resolvin E_1
RvE_2	Resolvin E_2
SAA	Serum amyloid A
SEM	Structural equation modelling
SOC	Suppressors of cytokine signalling
SHP2	Protein-tyrosine phosphatase
STAT	Signal Transducer and Activator of Transcription
STEMI	ST-elevation myocardial infarction
NSTEMI	Not ST-elevation myocardial infarction
TLR	Toll like receptor
TMB	3,3',5,5'-Tetramethyl-benzidine
TNF-α	Tumour Necrosis Factor α
TGF-beta	Transforming growth factor-beta
TX	Thromboxane
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
VLA4	Very-late antigen 4
WHO	World Health Organizatio

WT	Wild type
7TM	7 Transmembrane

CHAPTER 1: INTRODUCTION

Coronary Heart Diseases (CHD), diseases of the blood vessels supplying the heart muscle, are one of the leading causes of death and disability in the world. According to the WHO (World Health Organization), 7.3 million people died from CHD in 2008 (Mathers and Loncar 2006). The effects of CHD are usually attributable to acute myocardial ischemia-reperfusion injury (IRI) (Mathers and Loncar 2006).

In patients affected by acute myocardial infarct (AMI) the most effective therapeutic intervention for reducing IRI and limiting heart damage is timely myocardial reperfusion using either thrombolytic therapy or primary percutaneous coronary intervention (PPCI). However, reperfusion is a double-edged sword. It can itself induce further cardiomyocyte death, a phenomenon known as myocardial reperfusion injury. One of the main characteristics of reperfusion injury is a marked prolongation of the inflammatory response initiated during ischemia. For this reason, in myocardial ischemia-reperfusion injury, inflammation is a prerequisite for healing (Moens, Claeys et al. 2005) but it can paradoxically also extend tissue injury, hence it needs to be modulated. A greater understanding of this disease and how inflammation is involved should lead to more effective treatments.

It is now appreciated that the termination of an acute inflammatory response is not just a passive process but relies on the active involvement of pro-resolving mediators, which constrain the host reaction avoiding over-shooting and self-inflicted tissue damage (Serhan and Savill 2005, Serhan, Brain et al. 2007). Following this idea, appreciation of the mediators operative in the area of resolution of inflammation for AMI is emerging, and these include the protein Annexin A1

(AnxA1; (Perretti and D'Acquisto 2009) and its receptor, the G protein coupled receptor termed formyl peptide receptor type 2 (FPR2 (Ye, Boulay et al. 2009)), though as yet there is little in-vivo evidence for their modulatory functions.

AnxA1 is able to influence polymorphonuclear leukocyte (PMN) and macrophage biology.

This mediator is a potent inhibitor of PMN interaction with the vascular endothelium (Hayhoe, Kamal et al. 2006) and promotes PMN apoptosis (Solito, Kamal et al. 2003). In addition, AnxA1 promotes efferocytosis of dead/dying PMNs by macrophages (Scannell, Flanagan et al. 2007), which is central to the restoration of tissue homeostasis. The anti-inflammatory properties of this mediator converge onto FPR2/ALX, which will be extensively studied in this work using an experimental myocardial infarct model in mice. Indeed, human FPR2/ALX exhibits homology to 2 mouse genes, termed frp2 (74% of homology) and fpr3 (76% homology).

Here we investigated the role of the receptor FPR2 and its agonist in AMI, exploring also damage in secondary organs such as the lung and the kidney. The potential anti-inflammatory properties evoked by FPR2 activation appears to play an important role in affording protection in ischemia reperfusion injury and may lead to potential development of novel therapeutic targets.

1.1 INFLAMMATION

1.1.1 Overview

Inflammation (from latin, *inflammare*, to set on fire) is the body's first system of alarm signals that are used for the containment and elimination of microbial invaders after infection or injury (such us heat or cold exposure, ischemia/reperfusion injury, trauma, etc.). The body attempts to restore the tissue to its preinjury state. Uncontrolled inflammation has emerged as a pathophysiologic basis to many disease such us cardiovascular and neurodegenerative diseases and cancer progression. To better manage diagnosis, treatment and prevention of these diseases, multidisciplinary research is under way.

The first recorded observation of the inflammatory process was described by Aristotele (384-322 BC) as an imbalance of four humours: earth, fire, wet and cold. Next, at the time of Hippocrates (460-380 BC), inflammation was also called phlegmone', which translates loosely to "the burning thing". The Roman Cornelius Celsus (30 BC – 38 AD), in the first century, is credited as first documenting the four cardinal signs of inflammation: Rubor, Tumor, Calor and Dolor (redness, swelling, heat and pain)(Rocha e Silva 1978). In the next years the fifth of the cardinal signs of inflammation, the functio laesa (loss of function), was added by Galen (129-207 AD), even though the attribution is disputed. It has also been ascribed to Thomas Sydenham (1624-1689 AD) and Rudolph Virchow (1821-1902 AD)(Benaroyo 1994).

In 1908, Metchnikoff (1845-1916) described inflammation as a “defensive cellular response to pathogens, guided by the vessels rather than an aspect of the pathology itself” (Karnovsky 1981). This represented the first basic definition of inflammation. Subsequently the biological cell-based definition of inflammation was revolutionized by William Harvey (1578-1657), who first described the circulatory system in accurate details, and by the discoveries in microscopy and cell biology of the 19th century. The biochemical definition of inflammation was brought forward in 1974, by Rocha and Silva, who described inflammation as a “multi-mediated phenomenon, of a pattern type in which all mediators would come and go at the appropriate moment, increasing vascular permeability, attracting leukocyte, producing pain, local edema and necrosis” (Ryan and Majno 1977).

In 2012, the contemporary definition of inflammation is as follow:

“*Acute inflammation is the immediate defensive reaction of tissue to any injury, which may be caused by infection, chemicals or physical agents. It involves pain, heat, redness, swelling and loss of function of the affected part. Blood vessels near the site of injury are dilated, so that blood flow is locally increased. White blood cells enter the tissue and begin to engulf bacteria and other foreign particles. Similar cells from the tissues remove and consume the dead cells, sometimes with the production of pus, enabling the process of healing to commence. In certain circumstances healing does not occur and chronic inflammation ensues*”. Concise Medical Dictionary. Oxford University Press

1.1.2 The inflammatory response

The inflammatory response consists of an innate system of cellular and humoral responses following injury, infection or tissue stress or malfunction (Medzhitov 2008). The acute inflammatory response begins within seconds to minutes following injury. Tissue-resident macrophages upon recognition and activation by pathogens generate pro-inflammatory mediators (Ryan and Majno 1977, Nathan 2002). These include chemokines (Interleukin 8, IL-8), cytokines (Tumour Necrosis Factor α, TNF-α and Interleukin 1β, IL-1β), cell adhesion molecules (CAMs), lipid-derived eicosanoids (prostaglandins and leukotrienes) and vasoactive amines. This event leads to endothelial cell activation, stimulating the exposure of adhesion molecules on the luminal surface of the endothelium and promoting leukocyte migration from the circulation to the damaged sites (Ryan and Majno 1977).

Neutrophils, the most abundant leukocytes in the blood (Witko-Sarsat, Rieu et al. 2000), are the first to be recruited from the blood vessels to inflamed tissue. Their recruitment requires the canonical steps of rolling, activation, firm adhesion and transmigration (Witko-Sarsat, Rieu et al. 2000). The leukocyte interaction with the endothelium occurs mostly in the veins (39%) instead of the arteries (0.6%) (McEver and Cummings 1997); rolling is characterized by weak adhesion between neutrophils or monocytes and the endothelial cell wall. L-selectin, expressed on leukocytes, regulates the first step of cell rolling. Inflamed endothelial cells and platelets express E and P-selectin. P-selectin interacts with P-selectin glycoprotein ligand 1 (PSGL1), located at the tip of leukocyte microvilli (McEver and Cummings 1997). E-selectin binds to E-selectin ligand 1, which is highly homologous to the cysteine-rich FGF receptor located on neutrophil microvilli, and to the proteoglycan

CD44 (Hidalgo, Peired et al. 2007). The 'slow rolling' phase involves E-selectin and β2-integrins. Upon activation, integrins switch from the default low-affinity state to the high affinity-state (Jung, Ramos et al. 1998). The most important integrins involved in the process of cell adhesion are those of the β2 family such as, for example, lymphocyte function-associated antigen 1 (LFA1, αLβ2-integrin) - that binds ICAM-1 and ICAM-2 (cell adhesion molecules) and macrophage receptor 1 (MAC1, also known as CD11b/CD18, α4β1-integrin) that binds ICAM-1 and CD11c. There is also the ligand very-late antigen 4 (VLA4; of the β1 family) that binds ICAM-1 and promotes chemotaxis (Dunne, Ballantyne et al. 2002). Upon binding to their counter-ligand, integrins lead to "inside-out signaling"; in this cellular response, β2 integrins migrate from the cytosol to the cell surface and congregate in high-avidity patches that interact with the leukocyte cytoskeleton through cytosolic factors such as talin, α-actinin, kindlin and vinculin (Dustin and Springer 1989, Laudanna, Kim et al. 2002). The firm adhesion phase is followed by a shape change in migrating leukocytes and endothelial cells that allows the transmigration of the leukocytes through the endothelium, a process termed diapedesis. The integrin-mediated leukocyte transmigration also requires polarization of the cell body, formation of a lamellipodium at the leading edge and a uropod at the tail of the leukocyte. This process is mediated by endothelial PECAM-1 (Platelet endothelial cell adhesion molecule), JAMs (junctional adhesion molecule) and CD99 (Wehrle-Haller and Imhof 2002, Ridley, Schwartz et al. 2003). In the interstitial fluid, leukocytes followed by monocytes and lymphocytes, migrate along a chemotactic gradient towards the site of injury (Medzhitov 2008). Neutrophils release the toxic contain of their granules, composed by reactive oxygen and

nitrogen species, proteinase 3, cathepsin G and elastase, to kill the invading agent (Nathan 2006). The switch from PMN to mononuclear cells bridges the initial pro-inflammatory response with the activation of pro-resolution mediators involved in clearance, tissue remodeling and repair. Pro-inflammatory prostaglandins switch to resolvins, that initiate the tissues repair and lipoxins, able to inhibits neutrophils recruitment and promote monocytes infiltration. Monocytes are particularly important in this phase thanks to their ability to differentiate into professional antigen presenting cells (APC), such as M2 macrophages (Kantari, Pederzoli-Ribeil et al. 2008, Mills 2012) or dendritic cells that produce an anti-inflammatory environment (Bellingan, Caldwell et al. 1996, Schmidt, Nino-Castro et al. 2012). In the resolving phase leukocytes are removed either *via* the lymphatics or by apoptosis and phagocytic clearance, and the increased vascular permeability is reversed due to closure of the open inter-endothelial junctions. In both vascular and extravascular compartments, fibrin deposits are removed by plasminogen.

Following all these steps, the on-going acute inflammation response, which may last from 15 minutes to several hours or days, is terminated. If this response is not completely removed – or *resolved* - then chronic inflammation might arise leading to a variety of disorders. Examples include rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, multiple sclerosis, Crohn's disease, ulcerative colitis and some cancers (e.g., gall bladder carcinoma), acne vulgaris, chronic prostatitis, glomerulonephritis, psoriasis, pelvic inflammatory disease and vasculitis or myopathies. Also degenerative disease such as atherosclerosis, Alzheimer's and obesity are also characterized by the presence of a chronic inflammation process.

In summary, acute inflammation is a tissue reaction to injury characterized by dilation of blood vessels and consequent cells exudation acting to repair the damage. Chronic inflammation, might be defined as the persistence of acute inflammation or as the persistent engagement of innate and acquired immune response.

1.1.2 Lipid mediators in Acute Inflammation and Resolution

Inflammation triggers the production of different inflammatory mediators that have affects on the vasculature, on the recruitment of leukocytes or can alter the functionality of tissues and organs. Inflammatory mediators can be classified into seven group according to their biochemical properties: vasoactive amines, vasoactive peptides, fragment of complement components, lipids mediator, cytokines, chemokines and proteolytic enzymes (Medzhitov 2008). Vasoactive amines (histamine and serotonin) are released from mast cells and platelets degranulation (Spector and Willoughby, 1964). Vasoactive peptides can be stored in secretory vesicles (substance P) or generated by proteolytic processing of inactive precursors in the extracellular fluid (kinins, fibrinopeptide A or B and fibrin degradation products) (Gonzalez-Rey and Delgado 2005). Both vasoactive amines and peptide induce mast cells degranulation, an increase in vascular permeability and vasodilatation or constriction. The complement fragments, the anaphylatoxins (C3a, C4a and C5a), are produced by complement activation and they promote granulocyte and monocyte recruitment and mast cells degranulation, thereby affecting the vasculature (Haas and van Strijp 2007). Cytokines (e.g. TNFa, IL-6, IL-1) are produced by different cell types, especially macrophages and mast cells and they have different roles including leukocytes and endothelium activation. Chemokines (e.g. neutrophil chemoattractant KC, monocyte chemoattractant protein-1 MCP1), produced by many cells in response to inflammation control leukocytes chemotaxis towards the affected tissues. Proteolytic enzymes (elastin, cathepsins and matrix metalloproteinases) degrading the extracellular matrix

(ECM), are important in host defence, tissue remodelling and leukocytes migration (Medzhitov, 2008).

There are two classes of lipid mediators: eicosanoids and platelet-activating factors. Platelet-activating factors are generated by the acetylation of lysophosphatidic acid and activate several processes that occur during the inflammatory response, including leukocytes recruitment, vasodilatation and constriction, increase in vascular permeability and platelet activation (Yost, Weyrich et al. 2010). Eicosanoids are arachidonic acid-derived autacoids, locally acting substance that are rapidly synthesized in response to specific stimuli, and are involved in inflammation, cardiovascular and reproductive physiology.

Arachidonic acid (AA), an omega-6 PUFA (polyunsaturated fatty acid), is released from phospholipids that are present in the inner leaflet of cellular membranes by phospholipase A_2 (PLA_2) that hydrolysed the acyl ester bond. PLA_2 can be membrane-bound or secretory ($sPLA_2$) and soluble in the cytoplasm ($cPLA_2$). AA can also be synthesized from the essential fatty acid precursor linoleic acid, which can be obtained only from dietary sources. Next, arachidonic acid is metabolized to eicosanoids by cyclooxygenases (COX), lipoxygenase (LOX) or epoxygenase (cytP450) enzymes that dictate the class of eicosanoids generated (Figure 1A).

The COX pathway produces prostaglandins, prostacyclin and thromboxane. COX (also know as prostaglandin H), exists in two isoform: the constitutive COX1 and the inducible COX2. Each COX catalyses the cyclization of AA into prostaglandin G_2 (PG_2) that is reduced to PGH_2, the last unstable common mediator of this

pathway. Tissue-specific enzyme expression determines the tissues in which the various PGH_2 are produced. All prostaglandins share the same chemical structure characterized by 20-carbon carboxylic acid containing a cyclopentane ring and a 15-hydroxyl group, and are divided in three groups: PG_1, PG_2 and PG_3 depending on the number of double bonds in the molecule. PG_2 series is the direct product of AA, PG_1 series derived from the dihomo-Ɣ-linoleic acid (DHGLA) and the PG_3 one derive from eicosapentaenoic acid (EPA).

Prostaglandins are involved in inflammation and different physiological processes (Ricciotti and FitzGerald 2011) (Figure 1B). For example PGD_2 induces bronchoconstriction and controls the sleep function and PGE_2 is a vasodilatator, induces inflammatory cells activation and mucus protection. $PGF_{2\alpha}$ is involved in the reproductive physiology. Thromboxane (TXA_2) is produced in platelet by the thromboxane synthase and acts as vasoconstrictor and agonist of platelet aggregation. Prostacyclin (PGI_2) is the primary product of vascular endothelium function as vasodilator and inhibitor of platelet aggregation. It is clear that TXA_2 and PGI_2 are critical in the regulation of the blood pressure and thrombogenesis. An unbalance of TXA_2 and PGI_2 can lead to hypertension, ischemia, thrombosis and AMI.

The LOX pathway produces leukotrienes and lipoxins through the lipoxygenase (LOX) 5, 12 and 15, enzymes able to add a molecular oxygen into arachidonic acid. Leukotrienes are biosynthetised by the 5-LOX that convert the AA into 5 hydroperoxyyeicosatetraenoic acid (HPETE) and further into leukotriene A_4 (LTA_4). LTA_4 is then converted to either LTB_4 or LTC_4 depending on the cells type. LTB_4 is

produced in neutrophils, erythrocytes, macrophages and monocytes and leads to neutrophils chemotaxis, aggregation and transmigration and free radical and cytokines production. LTC_4, synthetized in mast cells, eosinophils and macrophages and it can be cleaved by peptidases to generates the cystenyl leukotrienes: LTD_4 and LTE_4. They cause vasoconstriction, bronchospasm and increase in vascular permeability. Lipoxins (LXA_4 and LXB_4) are important in resolution, decreasing neutrophil infiltration and transmigration, inhibiting eosinophil recruitment, stimulating vasodilatation through the synthesis of PGI2 and PGE2 and counteracting pro inflammatory mediators. They are synthesised by the 15-LOX and 5-LOX. Both cysteinyl leukotrienes and lipoxins are generated by transcellular biosynthesis between neutrophils and platelets or neutrophils and endothelial cells (Serhan 1997)(Figure 1B).

The cytP450 is involved in the production of epoxyeicosatetraenoic acids (EET). It is important in the regulation of smooth muscle cells, vascular tone and renal function.

Recently, two new families of bioactive mediators byosynthesized from omega-3 essential PUFAs have been discovered, and named the resolvins and the protectins (Kohli and Levy 2009).

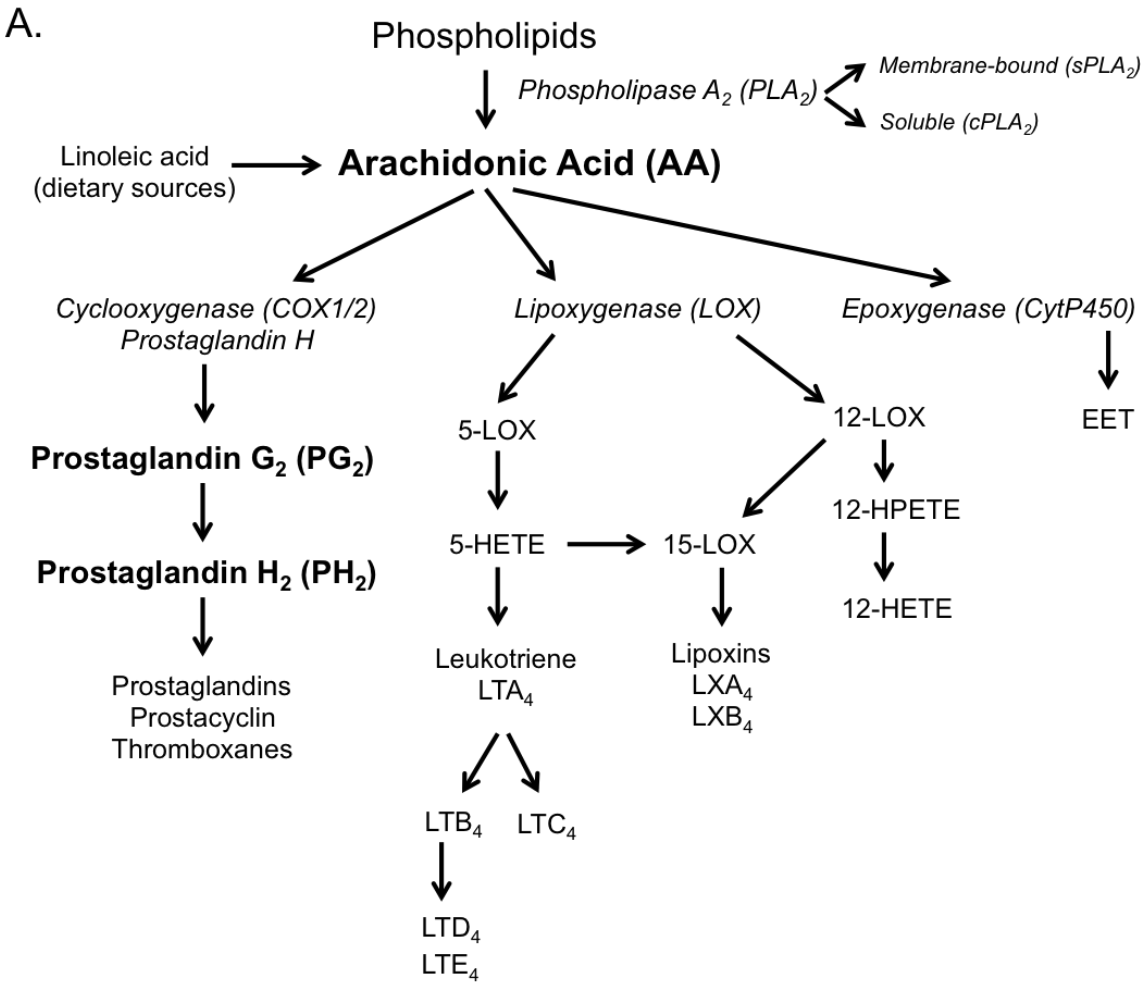
Resolvins (resolution phase interaction products) derived from EPA and docosahexaenoic acid (DHA) with two different chemicals structure: the E-series and D-series (Figure 2). Resolvin E1 and E2 are potent anti-inflammatory mediators (decrease neutrophils infiltration and TNF-α activity) thoughts EPA, 18-HEPE and 5-LOX. D-series resolvins have the same anti-inflammatory role and

synthesis but they first derive from DHA (Serhan, Hong et al. 2002). Aspirin increase their production (Oh, Vickery et al. 2011, Dalli, Winkler et al. 2013). Protectins are biosynthesized via a LOX mechanism that converts DHA to protectin D1, an anti inflammatory mediators that blocks T-cell migration and promotes T-cell apoptosis.

Research over the past 10 years has demonstrated the physiological benefits of increasing dietary consumption of omega-3 PUFAs by promoting the resolution of the inflammatory cycle. Moreover, considerable research supported cardiovascular benefits of consuming fish or fish oil containing the omega-3 eicosapentaenoic acid and docosahexaenoic acid. Both PUFAs reduce ex vivo platelet aggregation and are associated with lower risk of fatal cardiac events. DHA also modulates LDL and HDL particle sizes and decrease the risk of atrial fibrillation (Mozaffarian and Wu 2012). Interesting the American Heart Association recommends, after randomized control trials (RCTs), to eat 1g/day of EPA and DHA from oily fish or supplements to people with coronary heart disease. Consumption of the oily fish twice per week should also be used as prevention (<1g/day) (Breslow 2006). However, higher levels of omega-3 PUFA exert suppressive effects on the immune system (Simopoulos 2002). In line with that and considering also the dietary sources of omega-6, the omega-6/omega-3 fatty acid ratio became important in cardiovascular and other diseases.

Omega-6 fatty acids are found in vegetable oils, nuts and seeds. For example, linoleic acid is the primary dietary omega-6 PUFA. After consumption, LA is metabolized in γ-linolenic and dihomo-γ-linolenic acids and the latter is converted

in the most metabolically important omega-6 PUFA: the arachidonic acid. As said before, AA is the substrate for the production of a wide variety of eicosanoids: some proinflammatory and some anti-inflammatory (Harris, Mozaffarian et al. 2009). Excessive amounts of omega-6 PUFA and a very high omega-6/omega-3 ratio promote the pathogenesis of cardiovascular disease, cancer, and inflammatory and autoimmune diseases. On the other hand, as said before, a lower omega-6/omega-3 ratio (higher omega-3), exert immune-suppressive effects. In the prevention of cardiovascular disease, a ratio of 4/1 of omega6/3 is the preferred (70% decrease of mortality). A ratio of 2–3/1 is beneficial in inflammatory diseases (Simopoulos 2008).



B.

COX pathway	LOX pathway
PGD_2: bronchoconstriction PGE_2: vasodilator, edema TXA_2: vasoconstrictor, platelet aggregation PGI_2: vasodilator, inhibition platelet aggregation	LTB_4: chemotaxis, cytokine production LTD_4 and LTE_4: vasoconstrictor, bronchospasm, increase vascular permeability

Figure 1: The arachidonic acid pathway

(A) The arachidonic acid released from the phospholipid by the enzyme PLA_2 is the substrate for cyclooxygenase, lipoxygenase and epoxygenase. The product of these reactions proceeds through a further sequence of enzymatic reactions to produce prostaglandin, prostacyclin and thromboxane (COX pathway), leukotrienes and lipoxins (LOX pathway) or epoxyeicosatetraenoic acids (EET) (CytP450 pathway). (B) Example of metabolites derived from the COX and LOX pathways with their effects of human physiology.

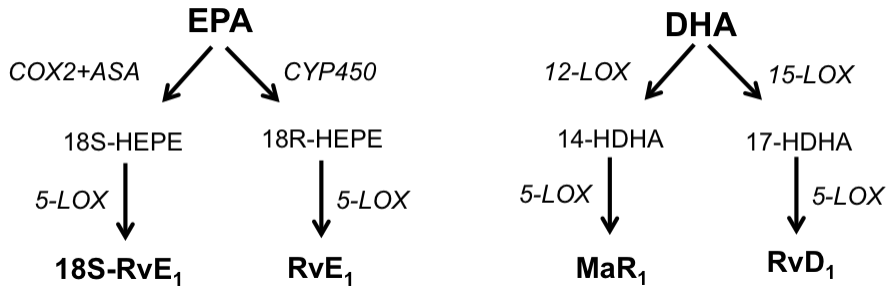


Figure 2: The resolvins pathway

Two of the major types of omega-3 fatty acids are the eicosapentaenoic acid (EPA) and the docosahexaenoic acid (DHA) precursors, thought enzymatic reactions, for potent anti-inflammatory lipids called resolvins (Rvs) and maresins (MaR).

1.1.3 “The beginning programs the end”

The usual outcome of the acute inflammatory response is resolution and repair of tissue damage, and it can be divided into three phases: onset, transition and resolution (Serhan and Savill 2005). If resolution is not completed acute inflammation may became chronic. In the initial onset phase there is a release of inflammatory mediators from local tissues and resident cells. This induces leukocyte migration to the site of inflammation, which peaks during the transition phase (Serhan and Savill 2005). In the early phase pro-inflammatory mediators are released, which initiate the acute-phase of the inflammatory response. However, this is counterbalanced by endogenous anti-inflammatory signals (Serhan and Savill 2005) such as corticosterone (or cortisol in man), which serve to temper the severity and limit the duration of the early onset phase.

Besides cortisol and corticosterone, during the progression of the inflammatory response some “stop signals” are activated. They are important to prevent further leukocyte traffic into the tissue (Serhan and Savill 2005). Examples of stop signals are the process of apoptosis and down regulation of pro-inflammatory molecules, such as leukotrienes, or desensitization of receptors by high concentration of ligands. This phase is also characterized by the release of anti-inflammatory molecules such us lipoxins, resolvins (Serhan 2008), IL-10 (Sato, Ohshima et al. 1999) and prostaglandins (PGs) of the D series (Luster, Alon et al. 2005).

Along this line is the concept that in the resolution phase is not just a passive process. The balance of pro-resolving mediators (Lawrence, Willoughby et al.

2002) and pathways outweighs the pro-inflammatory, resulting in suppression of pro-inflammatory gene expression and cell trafficking, clearance of pathogens and apoptotic cells and recovery of homeostasis (Serhan 2004, Serhan and Savill 2005, Serhan, Brain et al. 2007), which is lost when this balance is broken. Pro-resolving endogenous mediators assure temporal and spatial containment of the host reaction (Serhan and Savill 2005, Nathan 2006).

LXA_4 and AnxA1, together with the receptor, formyl peptide receptor type 2 (acronym FPR2/ALX), represent some of the endogenous anti-inflammatory mediators involved in the resolution process (Perretti and D'Acquisto 2009, Dufton and Perretti 2010). Moreover, although they are very different in their chemical nature, synthetic modalities (produced on demand or stored in cell sources), catabolism and half-lives, they share similar pro-resolving properties upon binding to the same receptor (Dufton, Hannon et al. 2010). LXA_4 and AnxA1 are able to stop the process of leukocyte migration (Perretti and D'Acquisto 2009), promote macrophage phagocytosis of infective agents and remove apoptotic leukocytes (Godson, Mitchell et al. 2000, Oliani, Christian et al. 2000). AnxA1 is also able to convey some inhibitory signals to mast cells (Oliani, Christian et al. 2000) which are sensitive to fMLF and involved in the pro-inflammatory phase.

1.2 ACUTE MYOCARDIAL INFARCT (AMI)

1.2.1 AMI aetiology and pathogenesis

Ischemic heart disease, stemming from coronary artery disease and Acute Myocardial Infarction (AMI), is the largest single cause of death worldwide (Murray and Lopez 1997, Bell and Yellon 2011).

Acute myocardial infarction is an irreversible myocardial injury resulting in necrosis of a portion of myocardium bigger then 1 cm causing a reduction of its function. Several studies have recognized the high impact that AMI has on patient morbidity and mortality through the development of heart failure and arrhythmias (Moens, Claeys et al. 2005).

The necrosis is generally endocardium-based, secondary to occlusion of an epicardial artery and located around the occluded vessel with a consequent lack of oxygen supply (Burke and Virmani 2007). The lack of blood flow is mainly due to acute thrombus overlying atherosclerotic plaque (Arbustini, Dal Bello et al. 1999). Atheroma or other obstructions can also be the cause. In the majority of patients, there is obstructive coronary disease at angiography. Hearts usually have a left coronary dominance (15% of the population). Left main coronary artery occlusion results in an anterolateral infarct, whereas occlusion of the left anterior descending coronary artery causes are limited to the anterior wall. In hearts with a right coronary dominance (with the right artery supplying the posterior descending branch), a right coronary artery occlusion causes an inferior infarct. However, unusual patterns of vessels supplying of the heart may also result in unexpected areas of infarct.

Non-reperfusion myocardial infarct, characterized by a permanent occlusion of the blood flow, also exists.

There are many risk factors that predispose to coronary artery disease. These include genetic polymorphisms involved in atherogenesis, coagulation, lipid metabolism, inflammation and in the renin-angiotensis-aldosterone system (Shiffman, Rowland et al. 2006).

The symptoms are chest pain, which may radiate to the arm, sweating, nausea and chest tightness or pressure. The diagnosis is based on laboratory findings of necrosis of the myocardium, which causes leaking of myocardial enzymes, such us troponin, into the circulation. Acute infarcts are divided into ST-elevation myocardial infarction (STEMI) and non ST-elevation infarction (non-STEMI). STEMI is the result of blockage of a coronary artery with large amount of cardiac enzyme in the serum and Q waves in the electrocardiogram (ECG). Modest elevation of cardiac enzymes and small or patchy areas of necrosis characterize the non-STEMI infarct, often overlapped with the acute coronary syndrome unstable angina.

1.2.2 The contribution of inflammation to AMI

AMI is characterized by ischemia and subsequent reperfusion of the myocardium. Rapid restoration of blood flow within the first 2-3 h from onset of chest pain (Gersh, Stone et al. 2005) is essential to recover the affected area of the myocardium (Fu, Goodman et al. 2001). This reperfusion phase, together with the ischemic and post-ischemic inflammatory response (Frangogiannis, Smith et al. 2002), enhances structural and functional recovery of the heart, preserving left ventricular function, and improves survival, preventing the onset of heart failure (Di Napoli, Taccardi et al. 2002). However, it is a paradox that reperfusion itself is also responsible for myocardial damage. Reperfusion is characterized by ROS release (Kumar, Okuda et al. 1990) and intracellular accumulation of calcium (Coetzee and Opie 1992) leading to mitochondrial depolarization and breakdown (Lichtig and Brooks 1975), ATP depletion, contraction-band necrosis (Rodriguez-Sinovas, Abdallah et al. 2007) and an extended and increased inflammatory process initiated within the ischemia phase (Lefer and Granger 2000, Poon, Ward et al. 2001). Inflammatory processes including cytokine production, complement activation (Hill and Ward 1971) and tissue PMN infiltration (Romson, Hook et al. 1983, Carden and Granger 2000) play a major role in the extension of myocardial injury. At 12 hours after the onset of the chest pain the myocardium is characterized by hypereosinophilia leading to endomyocardial fibrosis, without the presence of the primary causes of eosinophilia (Ogbogu, Rosing et al. 2007, Gurgun, Tuluce et al. 2011), followed by neutrophil infiltration at 24h. Macrophages begin to appear by day 2-3 post-ischemia.

It is clear that inflammation, in acute myocardial infarct, is a prerequisite for healing (Richard, Murry et al. 1995) but it can paradoxically also extend myocardial injury leading to the phenomenon called “lethal reperfusion injury”. The infarcted heart displays signs of inflammation that, unless resolved, lead to long-term tissue damage.

It is for this reason that emerging therapeutics strategies have been focused on the inflammatory response. Experimental animal studies with compounds that inhibits inflammation such as lipoxygenase inhibitors, LTB4 antagonists (Shappell, Taylor et al. 1990), free radical scavengers (Jolly, Kane et al. 1984), anti-neutrophil antibodies (Romson, Hook et al. 1983) or neutrophil depleting antimetabolites (Mullane, Read et al. 1984) have demonstrated statistical reduction of ischemia reperfusion injury. However, in some cases anti-inflammatory drugs have produced critical results in clinical investigations. For example, administration of corticosteroids was shown to decrease leukocyte infiltration and infarct size (Libby, Maroko et al. 1973, Versaci, Gaspardone et al. 2002) but also lead to ventricular arrhythmias and collagen deposition delaying the healing process (Roberts, DeMello et al. 1976, Kloner, Fishbein et al. 1978, Solomon, Schneeweiss et al. 2004, Hippisley-Cox and Coupland 2005, Nussmeier, Whelton et al. 2005).

Thus, there is a need for a better understanding of the mediators involved in the inflammatory response after myocardial infarct in order to develop more specific interventions that could mitigate inflammatory injury during early reperfusion without interfering with myocardial healing. It is now appreciated that the acute inflammatory response is not simply a passive process, but relies on the active

involvement of pro-resolving mediators which assure the containment of the host reaction (Serhan and Savill 2005, Serhan, Brain et al. 2007), in line with the concept that “the beginning programs the end”, recently put forward (Serhan and Savill 2005). Hence, promoting endogenous anti-inflammatory mediators may represent an alternative approach to the treatment of the ischemic diseases; where the pro-resolving mediators of the inflammatory response can be targets to reduce myocardial ischemia and reperfusion injury (Steffens, Montecucco et al. 2009).

1.2.3 The cardioprotective IL-6/JAK/STAT3 pathway

Plasma levels of pro-inflammatory cytokines, such us IL-6, TNF-α and IL-1β are elevated after myocardial infarct (Munkvad, Gram et al. 1991, Guillen, Blanes et al. 1995, Manginas, Bei et al. 2005). These cytokines play an important role in the modulation of the inflammatory response after AMI and are associated with left ventricle remodelling (Ono, Matsumori et al. 1999, Hayashidani, Tsutsui et al. 2003, Dewald, Zymek et al. 2005). Among these cytokines IL-6 plays an important role in AMI.

IL-6 is only one of the members of a large IL-6 family that includes leukaemia inhibitory factor (LIF), cardiotrophin 1 (CT-1), oncostatin M (OSM), cilliary neurotrophic factor (CNTF), IL-11 and IL-27 which share the common receptor subunit gp130 for signalling.

IL-6 secreted by macrophages, T cells (Hagiwara, Abbasi et al. 1995) and cardiac neutrophils (Neumann, Ott et al. 1995) levels are upregulated in both plasma and heart after AMI (Deten, Volz et al. 2002, Kaminski, Kozuch et al. 2009) and are also prognostic markers for morbidity and mortality. Administration of IL-6 antagonists affords beneficial effects on the development of left ventricular remodelling after AMI (Kobara, Noda et al. 2010). IL-6 activates cells by binding to the α-subunits of the membrane-bound receptor (mIL-6R) and the soluble form sIL-6R and inducing homodimerization of signal-transducing gp130 molecules (Taga, Hibi et al. 1989). In the infarcted myocardium, the major IL-6R expressing cells are neutrophils and monocytes. Moreover, plasma levels of sIL-6R in patients with AMI

are elevated, leading to activation of gp130-expressing cells including cardiac myocytes (Kimura, Kanda et al. 2000).

Homodimerization of the gp130 receptor β-subunits or heterodimerization of gp130 with either the LIF or OSM receptor subunits leads to the activation of intracellular signalling cascades including the Janus kinase/signal transducer and activator of transcription (JAK/STAT), Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase pathways (Fischer and Hilfiker-Kleiner 2008). The protein-tyrosine phosphatase/extracellular-signal-regulated kinases (SHP2/ERK) and STAT pathways play an important roles in cardiac development, hypertrophy, protection and remodeling in response to physiological and pathophysiological stimuli (Fischer and Hilfiker-Kleiner 2008).

The contribution of ERK1/2, PI3K/Akt and JAK/STAT signalling to cardiomyocyte hypertrophy and survival is complex. ERK1/2 and PI3K play an important role in regulating hypertrophic gene expression and aspects of myofilament remodelling, whereas JAK/STAT activation may be important in myofilament organization (Fischer and Hilfiker-Kleiner 2008). However, although new evidence suggests that ERK1/2 and PI3K activation by IL-6 are sufficient for cardioprotective activity (Bolli, Stein et al. 2011), the JAK/STAT3 pathway, with a particular role for STAT3, is the most recognized for its beneficial role in AMI (Negoro, Kunisada et al. 2000).

Four JAKs and seven STATs have been identified. JAK1, JAK2 and TYK2 (Tyrosine Kinase 2) are present in cardiomyocytes, JAK3 in the thymus (Pan, Fukuda et al. 1999). All seven STATs (STAT1, STAT2 STAT3, STAT4, STAT5A STAT5B and STAT6) are expressed in, endothelial cells smooth muscle cells,

cardiac fibroblast and cardiomyocytes (Xuan, Guo et al. 2001). STAT3 is the more likely to be involved in cardioprotection, especially after ischemia reperfusion injury. STAT3 activation has positive effects on infarct size, since it leads to anti-apoptotic activity and up-regulates the COX-2 enzyme (Xuan, Guo et al. 2003), which confers protection. STAT3 also induces heme oxygenase-1, which has been recognized as a major cardioprotective and anti-apoptotic enzyme (Vulapalli, Chen et al. 2002). In addition, various anti-apoptotic proteins, involved in the mitochondria-mediated pathway of apoptosis, are known to be transcriptionally regulated by STAT1/3 (Fujio, Kunisada et al. 1997, Haga, Terui et al. 2003).

Upon activation, STATs are also able to bind to the promoter region of SOC genes (suppressors of cytokine signalling) and up-regulate the transcription of target genes (Cooney 2002). Importantly, SOC3 down regulates IL-6 (Croker, Krebs et al. 2003) and modulate its ability to increase infiltrated cells that represent an exacerbating factor in left ventricle remodelling after AMI. Indeed, IL-6 induces intracellular adhesion molecule-1, resulting in the adhesion of neutrophils and increase in tissues myeloperoxidale (MPO) levels, and MCP1 expression and monocytes infiltration. Recent data suggest that IL-6/gp130 signalling through STAT3 might also mediate the anti-inflammatory effects of IL-10 (Williams, Sarma et al. 2007).

A recent publication (Pupjalis, Goetsch et al. 2011) showed that the AnxA1 peptide Ac2-26, released by apoptotic cells, is able to activate the JAK/pTyrSTAT3/SOCs3 signalling pathway reducing IL-6 signalling and release of TNFα from endotoxin-challenged monocytes. The FPR2/ALX agonist WKYMVm can promote STAT3

serine, but not tyrosine, phosphorylation via ERK activation, blocking hydrogen peroxide-induced STAT3 activity, without concomitant JAK activation (Jo, Lee et al. 2004). This is in line with the ability of chemokine and other agonists to induce the JAK/STAT signalling binding to seven-transmembrane G-protein-coupled receptors (Pelletier, Duhamel et al. 2003, Soriano, Serrano et al. 2003, Wu, Lo et al. 2003).

1.2.4 Current treatment for AMI

Several approaches are being investigated for the prevention of cardiovascular diseases. Current therapies are antiplatelet agents, anticoagulants, vasodilators, diuretics, calcium-channel blockers, beta-blockers, angiotensin-converting enzyme (ACE) inhibitors and cardiac glycosides (Ryan, Antman et al. 1999, Siddiqui, Tandon et al. 2001, Braunwald 2012). However, there remains no effective therapeutic agent for preventing lethal myocardial reperfusion injury.

During acute myocardial infarct the treatment is aimed to restore the balance between the oxygen supply and the demand to prevent further ischemia and the prevention and treatment of complications. Pain relief is also considered. According to the 2013 guidelines from the American College of Cardiology Foundation/American Heart Association (ACCF/AHA), the first approach is the use of aspirin to inhibit thrombolosis (Hennekens and Dalen 2013) and the supplement of oxygen (to maintain oxygen saturation >90%) to prevent hypoxemia. Then, STEMI and NSTEMI patients are treated in different way.

Patients with STEMI are treated with thrombolysis or primary percutaneous transluminal coronary angioplasty (PTCA). NSTEMI patients receive anti-ischemic therapy (Antman, Anbe et al. 2004).

Beta-adrenergic blockers, like metoprolol and atenolol, are a benefit for both. They reduce the rates of reinfarction and recurrent ischemia. ACE inhibitors are useful for long-term therapy: they reduce the mortality rates after myocardial infarction. A

combination of beta-adrenergic blockers and ACE inhibitors improve the balance between myocardial oxygen supply and demand and limit infarct size.

Calcium channel blockers such as diltiazem and verapamil are used, in the absence of pulmonary edema, in patients where beta-adrenergic blockers are contraindicated. Recent studies showed that L-carnitine is able to limit the infarct size and to stabilize the cardiomyocyte membrane. L-carnitine reduces the mortality (27%) and ventricular arrhythmias (65%) (DiNicolantonio, Lavie et al. 2013).

In all cases morphine sulfate may be administered to relieve pain and anxiety.

Even if inflammation play a major role during AMI and during the no-reflow phenomenon where good myocardial perfusion is not achieved despite drug treatments, only the anti-inflammatory drugs aspirin cited above is used in coronary artery diseases. The effect of aspirin is due to an irreversible inhibition of COX-1 and TXA_2, a platelet aggregation factor, and to a reduction of the C-reactive protein, a clinical biomarker of risk of myocardial infarction (Ridker, Cushman et al. 1997). Inhibition of COX-2 in macrophages has been also suggested as another potential mechanism (Baker, Hall et al. 1999).

However, non-steroidal anti-inflammatory drugs (NSAIDs) are associated with an increased risk of reinfarction, hypertension, heart failure, and myocardial rupture (Antman, Hand et al. 2008). Diclofenac and other NSAIDs, with the exception of

Naproxen, increase the risk of cardiovascular events in patients with established CHD (Schjerning Olsen, Fosbøl et al. 2011).

For these reasons there is need for a better understanding of the role(s) of inflammation during reperfusion injury and a development of novel therapeutic approaches to the treatment of AMI.

1.2.5 Experimental models of AMI: relevance to humans

Animal models of myocardial ischemia reperfusion mimic the clinical scenario where a period of ischemia is followed by the restoration of myocardial blood flow. U-shaped constrictors, ring-shaped hydraulic occluders or intracoronary balloon catheters are used in large animals (pigs, dogs). In mice, two methods are used to induce myocardial ischemia. One is to create cryo-infarction using a cryo-pen on the surface of the heart. This technique is somewhat inaccurate as the ischemia is not guaranteed and it leads to side effects that are not related to myocardial infarction (Atkins, Hueman et al. 1999, Kolk, Meyberg et al. 2009). The second method consists in ligation of the left anterior descending coronary artery (LADCA) (Michael, Entman et al. 1995) and has several advantages. The surgery is extremely fast, with a duration ranging only from 3 to 5 minutes. By occluding the LADCA, the ischemia can be detected immediately as no further blood flow is permitted in the left ventricle, while the surrounding myocardial tissue is almost unaffected. It allows induction of myocardial infarctions of varying degrees and long-term survival as needed (Michael, Entman et al. 1995). The mortality within the first 24 hours after surgery is 37 to 50%. Among the causes of death, at the beginning the main factors are related to the surgical procedure and they are pneumothorax and respiratory depression (Klocke, Tian et al. 2007). However, 65% of deaths happens between 1.5 and 9 hours after LADCA occlusion and are caused by arrhythmias like ventricular tachycardia and ventricular fibrillation (Opitz, Mitchell et al. 1995).

The LADCA model is preferred because it is generally accepted as the model that better shares the clinical and pathological features of AMI. Moreover, this protocol

allows comparative and quantitative studies on the pathobiological and pathophysiological aspects occurring in infarction-related myocardial ischemia. The studies of the mechanisms involved in coronary artery occlusion and reperfusion are essential to develop and optimize future treatment for this pathology.

Here, the procedure of ischaemia and reperfusion is performed as described previously (Di Filippo, Rossi et al. 2004). Briefly, under anesthesia (Ketamine 100mg/Kg and Xilazina 16mg/Kg) a midline cervical incision separating the skin, muscle and tissue covering the trachea is performed. Subsequently a hole is made between two cartridge rings below the glottis of the trachea - of mice kept at a body temperature by a homeothermic blanket – allowing cannulation to maintain artificial ventilation. The tidal volume of the respirator is set a 1.0 ml/min, with the rate set at 110 strokes/min, and supplemented with 100% oxygen with no evidence of lungs or cardiovascular injury (Figure 3).

Figure 3: Photograph of open-chest mouse with trachea canulation.

After an equilibration period of 10 min, the mouse is turned on its right side and a left thoracotomy is performed (between the fourth and the fifth ribs ~3 mm from the sternum) and the pericardium is removed to expose the heart. Subsequently a silk ligature (7-0) is placed around the LADCA, located between the pulmonary artery and the left auricle, for 30 minutes. Both ends of the ligature around the coronary artery are threaded through a small polythene button placed in contact with the heart. Coronary artery occlusion is achieved by applying tension to it and clamping the ligature against the button with a rubber-sheathed artery clip. After the 2h hours of reperfusion, blood and tissues are harvested and used for further analysis.

Mice have a different septal coronary artery (Salto-Tellez, Yung Lim et al. 2004, Kumar, Hacker et al. 2005) but the ligation of the left coronary artery as it emerges from under the left atrium results in reproducible large infarctions (Salto-Tellez, Yung Lim et al. 2004, Kumar, Hacker et al. 2005) (Figure 4). The origin of the left coronary artery from the aorta up to 1-2 mm posterior to the left atrium is identical between C57BL/6 mice (Salto-Tellez, Yung Lim et al. 2004).

The infarct size resulting from the coronary occlusion can be up to the 65% of the area perfused from the coronary (Pfeffer, Pfeffer et al. 1979). When the occlusion is too close to the origin, with a septal artery involvement, the infatct size can be higher than 65% with a 100% of mortality (Pfeffer, Pfeffer et al. 1985).

Figure 4: Photograph of open-chest mouse with silk ligature on left anterior descending coronary artery.

The left anterior coronary artery of the mice originates between the pulmonary artery sulcus and the left atrium. The proximal region is intramyocardial, returning to the surface after 3 to 4 mm.

1.3 FORMYL PEPTIDE RECETOR 2

1.3.1 Overview of the Formyl Peptide Receptor family

G protein coupled receptors (GPCRs) are the largest group of receptors coded by the human genome. These seven transmembrane (7TM) receptors are involved in a variety of biological response like visual signal transduction, mood and behavioural regulation, olfactory modulation and control of cardiac function. Since they also transduce the action of chemokines and other inflammatory signals they are very important in the regulation of the body's defence system. For example, GPCR signalling is required for leukocyte transmigration into the inflamed area. They ensure appropriate arrival of the leukocyte at the site of infection where they are capable to kill, engulf and digest xenobiotics or infective agents (Yona, Lin et al. 2010). One family of GPCRs involved in the inflammatory response is the formyl peptide receptor family (FPRs).

Schiffman, in 1975, showed that synthetic N-formyl peptides, particularly the N-formyl-methionyl-phenylalanine (fMLF) derivatives, were potent neutrophil chemoattractants. The discovery that N-formyl peptides could stimulate chemotaxis at very low concentrations (Schiffmann, Corcoran et al. 1975, Zigmond 1977) provided strong evidence for the presence of a functional receptor for these molecules. Indeed, the human formyl peptide receptor (FPR) was subsequently defined pharmacologically as a high affinity-binding site on the surface of neutrophils for the peptide fMLF by Showell (1976) and Freer (1980, 1982). fMLF is the smallest formyl peptide that display full agonist activities and it is able to

stimulate chemotaxis, lysosomal enzyme release and superoxide generation (Schiffmann, Corcoran et al. 1975, Showell, Freer et al. 1976).

The human FPRs were first defined using radioisotope- and fluorescence-labeled peptide ligands. In 1977 Aswanikumar and William demonstrated that neutrophils express specific and saturable binding sites for fMLP (Williams, Snyderman et al. 1977). In 1979 Niedel demonstrated that fMLP retained full biological activity in human neutrophil chemotaxis assays. He also observed fMLP internalization and aggregation suggesting existence of a phenomenon of receptor binding followed by a receptor-mediated uptake of the ligand. Vitkauskas in 1980 and Donabedian and Gallin in 1981 showed deactivation and the internalization of the fMLP receptor. These approaches paved the way for the discovery and characterization of the neutrophil FPR, which was cloned by François Boulay in 1980 (Ye, Boulay et al. 2009). FPR (or FPR1 according to more recent nomenclature) is a glycoprotein of 57-70 kDa formed by a polypeptide of 350 aminoacids assembled as 7 hydrophobic trans-membrane domains. It maintains a three-dimensional structure by the presence of ionic bridges between the positively charged amino acids arginine and lysine and the negative charges of phosphates (Niedel and Cuatrecasas 1980, Dolmatch and Niedel 1983, Niedel and Dolmatch 1983, Allen, Jesaitis et al. 1986). Throughout the 1980s the FPR receptor was characterised as a pertussis toxin sensitive G-protein coupled receptor (GPCR) (Bokoch and Gilman 1984, Lad, Glovsky et al. 1985, Lad, Olson et al. 1985), which means it is coupled with to a G_i protein (Gierschik, Sidiropoulos et al. 1989, Uhing, Gettys et al. 1992, Lavigne, Murphy et al. 2002).

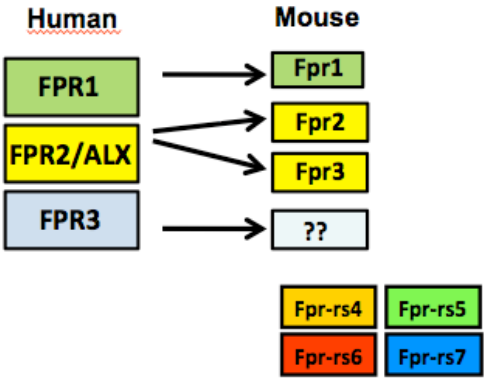
In 1992, several laboratories reported the identification of a 7TM receptor that shared significant sequence homology to human FPR (Bao, Gerard et al. 1992, Murphy, Ozcelik et al. 1992, Perez, Holmes et al. 1992, Ye, Cavanagh et al. 1992): the related FPR2/ALX receptor was therefore identified. The human pertussis toxin sensitive GPCR family of FPRs was completed (Boulay, Tardif et al. 1990) with three genes encoding FPR1, FPR2/ALX and FPR3 clustered on chromosome 19q13.3-19q13.4 (Ye, Boulay et al. 2009), whilst the murine Fpr gene family is located on chromosome 17 and consists of seven members. Human FPR1 has a direct orthologue in mouse Fpr, termed Fpr1 (76% of homology), with high affinity for N-formylated peptides (He, Liao et al. 2013). Human ALX/FPR2 exhibits homology to 2 mouse genes, termed frp2 (74% of homology) and fpr3 (76% homology) (Figure 5). Fpr2 binds the synthetic FPR2/ALX agonists WKYMVm, compound 43 and Quin-C1 (He, Liao et al. 2013). Other related murine genes, designated from fpr-rs3 to fpr-rs7, have also been identified but remain orphan receptors (Ye, Boulay et al. 2009).

FPR3, a 7TM receptor with 352 amino acids, shares 56% homology with human FPR1 (Ye, Boulay et al. 2009) although it is not activated by fMLP. FPR3 has no current orthologues in the mouse and has only one high affinity endogenous ligand, the heme-binding derived F2L peptide (Migeotte, Riboldi et al. 2005).

FPR1 and FPR2/ALX receptors are expressed on PMN and mononuclear cells, both myeloid and lymphoid (Schiffmann, Corcoran et al. 1975, Showell, Freer et al. 1976, Aswanikumar, Corcoran et al. 1977, Zigmond 1977). The FPR3 receptor is not expressed in neutrophils (Murphy, Ozcelik et al. 1992) but is found on

monocytes and dendritic cells. Immature DC expresses FPR1, FPR2/ALX and FPR3, however only FPR3 is retained in mature cells (Yang, Chen et al. 2002, Migeotte, Riboldi et al. 2005, Devosse, Guillabert et al. 2009). FPR2/ALX expression remains unchanged during monocyte differentiation into macrophages (Ye, Boulay et al. 2009), even if with a limited function in resident tissue macrophages (Hashimoto, Murakami et al. 2007).

The distribution of the human FPR1 and FPR2/ALX receptor is also mirrored by their murine orthologues, suggesting that they might maintain physiological roles across the species (Fu, Karlsson et al. 2006). The FPR1 receptor has also been found, using immuno-histochemical methods, in several organs and tissues, including epithelial cells (Rotrosen, Malech et al. 1987), endocrine cells (including follicular cells of the thyroid and cortical cells of the adrenal gland), platelets, liver hepatocytes and Kupffer cells, gut epithelial cells, smooth muscle cells, brain, spinal cord and both sensory and motor neurons (Lacy, Jones et al. 1995, McCoy, Haviland et al. 1995, Becker, Forouhar et al. 1998, Le, Hu et al. 2000, Czapiga, Gao et al. 2005, Leoni, Alam et al. 2013). However, tissues and cellular distribution of the human FPR gene family members remains, for the moment, a partially explored field.



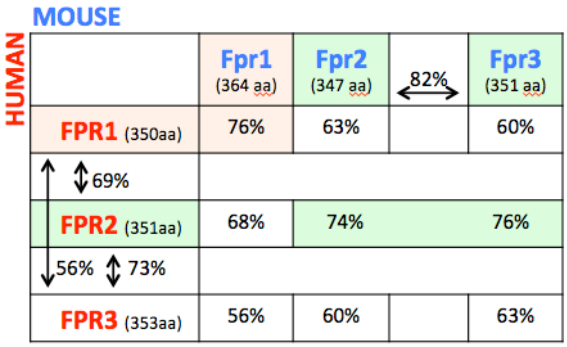


Figure 5: Schematic representation of the FPR receptors in human and mouse and Homology between human and mouse FPRs.

The FPR receptor family comprises seven homologues in mouse and three in humans with identified of highly conserved regions.

FPRs receptor signalling is regulated by agonist-induced internalization. Following binding with their agonists, the receptors undergo rapid phosphorylation in a concentration- and time-dependent manner (Ali, Richardson et al. 1993). This leads to conformational change to allow functional interaction with $G_i\alpha1$, $G_i\alpha2$ and $G_i\alpha3$ and association with $G_{0,}$ G_z and $G_{\alpha16}$ (Migeotte, Communi et al. 2006). This interaction triggers activation of a variety of signaling pathways (Figure 6) including fluxes in intracellular calcium concentration, activation of phospholipase C and D (PLC and PLD), PI3K and MAPK (Selvatici, Falzarano et al. 2006). PLC, involved in both PI3K and MAPK pathways *via* protein kinase (PKC), is responsible for receptor desensitization and internalization (Le, Wetzel et al. 2001). The MAPK pathway, in particular ERK 1/2, regulates chemokinesis (Wenzel-Seifert, Hurt et al. 1998). It has also been shown that PI3K, in neutrophils after interaction with fMLP, is able to regulate oxidative burst and actin relocation, essential for cell polarization (Dufton, Hannon et al. 2010).

As described above, FPR receptor signalling is followed by receptor desensitization (Ali, Richardson et al. 1999). For example, after activation of FPR1 with fMLP, the receptor reduces its responses to a secondary stimulation with the same agonist, a process referred to as homologous desensitization. FPR1 is also susceptible to heterologous desensitization, and has been shown to desensitize following activation of the complement receptor (C5aR), the interleukin-8 receptor (Ali, Richardson et al. 1999).

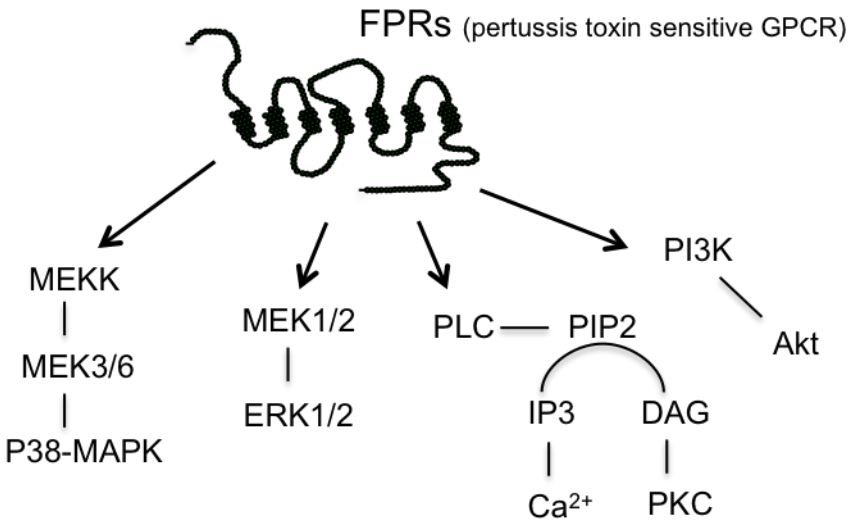


Figure 6: FPRs receptor signalling

Fprs agonists binding induce activation of different signalling pathways like MEKK/P38-MAPK, MEK-ERK1/2, PLC, PKC and Pi3K.

1.3.2 The Formyl Peptide Receptor 2 (FPR2/ALX)

The FPR2/ALX receptor was originally characterized as the low-affinity fMLF receptor (Murphy, Ozcelik et al. 1992, Ye, Cavanagh et al. 1992, Quehenberger, Prossnitz et al. 1993) and then identified also as the receptor for the LXA_4 (Fiore and Serhan 1995). FPR2/ALX is a 7TM receptor with 351 amino acids and share 69% identity with human FPR1 (Ye, Boulay et al. 2009). FPR2/ALX receptor has two different related murine genes, called Fpr2 and Fpr-rs2 (Hartt, Barish et al. 1999). Fpr2 showed closer sequence homology with FPR2/ALX, but Fpr3 showed more functional homology (Gao, Chen et al. 1998). Indeed mFpr3 is a low affinity receptor for fMLP (Hartt, Barish et al. 1999) and responds to several FRP2/ALX agonists like the amyloidogenic proteins serum amyloid A (SAA) (Liang, Wang et al. 2000) and amyloid $\beta_{(1-42)}$ (Le, Gong et al. 2001). Following the recent nomenclature guidelines from IUPHAR (Ye, Boulay et al. 2009) we can name the two genes, Fpr2 and Fpr3, as both being orthologues of human FPR2/ALX (Table 3). Mouse Fpr2 shares 81% of protein sequence with the mouse Fpr1 and 58% with the human FPR1 (Gao, Chen et al. 1998). Mouse Fpr2 and Fpr3 share the first exon, whereas the second exon that completes the transcription sequence differs between the two genes (Ye, Boulay et al. 2009).

FPR2/ALX expression is induced by both pro-inflammatory stimuli like TNF-α (Cui, Le et al. 2002) and pattern recognition receptors (Chen, Huang et al. 2009) as well as anti-inflammatory molecules, such as the glucocorticoids (Sawmynaden and Perretti 2006, Hashimoto, Murakami et al. 2007). FPR2/ALX is associated with host defence but, unlike FPR1, it seems to be involved in both pro and anti-

inflammatory processes. As an anti-inflammatory receptor it can induce deactivation, detachment and apoptosis of leukocytes, increase phagocytosis of apoptotic cells, and can also regulate COX-2 expression (El Kebir, Jozsef et al. 2007). FPR2 is also involved in the homeostasis and epithelial repair in the colon (Chen, Liu et al. 2013) and anti tumour activity (Liu, Chen et al. 2013). Most of the anti-inflammatory effects of FPR2/ALX are related to its endogenous ligands (Perretti, Chiang et al. 2002), like LXA_4, AnxA1, the neuroprotective peptide humanin and heme-binding protein-deriving peptide F2L.

Recent work from our lab, published during the completion of this thesis, indicates that FPR2/ALX follows ligand-biased pharmacology paradigms. Thus, it is activated by an array of different ligands resulting in different conformational changes and the ensuing intracellular responses: AnxA1 and LXA_4, but not the pro-inflammatory protein serum amyloid A, induce FPR2 homodimerization. This event led to a p38/MAPK-activated protein kinase/heat shock protein 27 signalling with generation of IL-10. The same study revealed that the pan-agonist peptide Ac2-26 induces FPR2/FPR1 heterodimerization and this activates a JNK-mediated proapoptotic path (Cooray, Gobbetti et al. 2013).

The versatility of FPR2/ALX is demonstrated by its interaction with the CC chemokine receptor 2 (CCR2) mediating monocyte-derived dentritic cells recruitment in allergic airway inflammation (Chen, Liu et al. 2013).

1.3.3 FPR2/ALX agonists

It is now known that FPR2/ALX can bind to a range of ligands, with putative pro and anti-inflammatory properties, and all of different natures, that is lipids, proteins and peptides. For example this receptor binds the glucocorticoids-modulated anti-inflammatory protein AnxA1 (Perretti, Getting et al. 2001 - FPRs KO mice and FPRs antagonists employed in the study) and the pro-inflammatory protein SAA (Su, Gong et al. 1999 - using HEK-FPRs transfected cells and antagonists). Other endogenous ligands include the mitochondrial peptides, the vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP)-27 (Cattaneo, Parisi et al. 2013 - using HEK-FPRs transfected cells and antagonists). FPR2 and in this case, FPR3, also bind the Hp(2-20) peptide derived from H. pylori, accelerating wound healing of gastric mucosa thought stimulation of eosinophil migration and vascular endothelial growth factor-a (VEGF-A) and transforming growth factor-beta (TGF-beta) production (Prevete, Rossi et al. 2013 - FPRs KO employed).

Screening of libraries of peptides has allowed the identification of various FPR2/ALX binding molecules, such as the synthetic peptide Trp-Lys-Tyr-Val_D-Met, also termed W-peptide or WKYMVm (He, Tan et al. 2000 - in vitro studies), that induces post-receptor Ca^{2+} flux, promotes cell chemotaxis (Le, Gong et al. 1999 - in vitro studies) and has therapeutic effects in ulcerative colitis (Kim, Kwon et al. 2013 - FPRs antagonists used). The small molecule pyrazolone-derived, the compound 43 (C43, designed by Amgen), represents another example of a synthetic FPR2/ALX agonist. C43 has anti-inflammatory activity by inhibiting fMLF

and IL-8 mediated chemotaxis in vitro and oedema formation in vivo (Burli, Xu et al. 2006 - in vitro studies).

The synthetic Quin-C1, a substituted quinazolinone, induces neutrophil calcium mobilization and chemotaxis (Nanamori, Cheng et al. 2004 - in vitro studies) and displays an anti-inflammatory effect in bleomycin-induced lung injury (He, Cheng et al. 2011).

Lipoxin A4 (LXA4) is part of the family of the lipoxins, eicosanoids generated from arachidonic acid via 5-, 12- and 15 LOX after interaction of leukocytes with platelets or epithelial cells (Chiang, Hurwitz et al. 2006, Chiang and Serhan 2006). The anti-inflammatory LXA_4 reduces PMN activity (Pouliot, Clish et al. 2000), promotes detachment of adherent leukocytes from mesenteric circulation (Gavins, Yona et al. 2003) and induces apoptosis and phagocytosis (Mitchell, Thomas et al. 2002) through FPR2/ALX (FPRs KO and receptor antagonists used). LXA4 also binds to other receptors, including a receptor shared with cysteinyl leukotriene receptor type 1 (CysLT1) (Chiang, Serhan et al. 2006) and the aryl hydrocarbon receptor (AhR) (Schaldach, Riby et al. 1999).

AnxA1 (Wallner, Mattaliano et al. 1986) is a 37kDa protein (consisting of 346 amino acids) and is one of the most studied anti-inflammatory FPR2/ALX agonists (Perretti, Getting et al. 2001, Gavins, Sawmynaden et al. 2005, Babbin, Lee et al. 2006, Hayhoe, Kamal et al. 2006 - (FPRs KO and receptor antagonists employed). Initially discovered as a mediator of glucocorticoid-induced inhibition of PLA_2 and called macrocortin or lipomodulin (Flower, 1979), these proteins were subsequently

shown to be identical and renamed under the unified name of lipocortin 1 (Pepinsky, Sinclair et al. 1986, Wallner, Mattaliano et al. 1986). Subsequent studies categorized it as a member of the annexin super-family (Pepinsky, Sinclair et al. 1986, Wallner, Mattaliano et al. 1986). The term annexin, from the Greek annex “hold together”, describes the ability of these proteins to bind negatively charged phospholipids in a calcium-dependent manner. AnxA1 is abundant in neutrophils, where it is mostly packaged within gelatinase granules (Perretti, Christian et al. 2000) but is also present in the cytosol (Ernst, Hoye et al. 1990). Upon neutrophil adhesion to endothelial cell (Perretti, Croxtall et al. 1996) or interaction with FPR2/ALX agonists (Brancaleone, Dalli et al. 2011) AnxA1 can be externalized and, by interacting with FPR2/ALX, is able to induce neutrophil detachment, apoptosis hence promoting the resolution of inflammation (Perretti and D'Acquisto 2009). Antiflammin 2 (AF2) is a nonapeptide corresponding to region 246-254 in the third repeat of the AnxA1 core (Miele, Cordella-Miele et al. 1988). Once the crystal structure of AnxA1 was resolved, it was noted that the AF2 sequence was exposed on the outer side of the protein (Rosengarth, Gerke et al. 2001). It was therefore proposed that this portion of the whole protein might be available to interact, in conjunction with the N-terminal region (Rosengarth and Luecke 2003), with specific molecular targets. Of interest, AF2 displays anti-inflammatory activities by inhibiting PMN activation, chemotaxis and adhesion to endothelial cells (Zouki, Ouellet et al. 2000). In some cases, these effects are through FPR2/ALX (Kamal, Hayhoe et al. 2006).

AnxA1 plays an anti-inflammatory role in arthritis (Patel, Kornerup et al. 2012), uveitis (Girol, Mimura et al. 2013), leukemic cells (Tsai, Lai et al. 2013), sepsis

(Gavins, Hughes et al. 2012), ulcerative colitis (Vong, Ferraz et al. 2012) and inflammatory pain modulation where can be a target for developing anesthesia and analgesic drugs (Chen, Lv et al. 2013).

Resolvin D1 (RvD1) is an omega-3 polyunsaturated fatty acids derived lipid mediators transcellular produced in human leukocytes and endothelial cells (Serhan, Hong et al. 2002) by sequential oxygenations by 15 LOX and 5-LOX. Omega-3 derivate are known to bestow protective clinical effects in the cardiovascular system and inflammatory disorders (De Caterina 2011). Specifically, RvD1 exhibit potent anti-inflammatory action in vivo (Hong, Gronert et al. 2003) and it limits neutrophil recruitment during acute inflammation through FPR2 and the orphan receptor G-protein-coupled receptor 32 (GPR32) (Norling, Dalli et al. 2012 - Receptor-specific antibodies and FPRs KO mice used).

As mentioned before, FPR2/ALX also binds pro-inflammatory molecules (as seen by both in vivo and in vitro studies using KO mice and receptors antagonists) and becomes involved in pro-inflammatory pathways. For example, binding the Serum Amyloid A (Liang, Wang et al. 2000), the peptide-derivative beta-amyloid-A ($A\beta_{42}$) (Yazawa, Yu et al. 2001) or the prion peptide PrP106-126 (Le, Yazawa et al. 2001), FPR2/ALX might be involved in amyloidosis (Munishkina and Fink 2007), atherosclerotic plaques (Wilson, Thompson et al. 2008), Alzheimer's disease (Cui, Le et al. 2002) and rheumatoid arthritis (Koga, Torigoshi et al. 2008, Nakamura 2008).

SAA is an inducible component of the acute phase and it is particularly abundant in chronic diseases: SAA serum levels increase up to 1000-fold after experimental or clinical inflammation (Kushner 1982). SAA is predominately secreted from the liver (Meek, Urieli-Shoval et al. 1994) and transported in the circulation primarily in association with high density lipoprotein (HDL) (Malle, Steinmetz et al. 1993). Besides systemic synthesis and delivery, SAA can also be produced at local inflammatory site (Vreugdenhil, Dentener et al. 1999) from endothelial cells, macrophages, adipocytes, and smooth muscle cells (Meek, Urieli-Shoval et al. 1994). SAA also interact with TLR4 (Sandri, Rodriguez et al. 2008) RAGE, TANIS, and CLA-1/hSR-B1 (Lakota, Mrak-Poljsak et al. 2013).

Through FPR2, SAA mediates a chemotactic response in human monocytes (Su, Gong et al. 1999) and is also able to induce neutrophil chemotaxis (Liang, Wang et al. 2000) and neutrophil adhesion to endothelial cells (Badolato, Wang et al. 1994). All these properties are associated with FPR2/ALX binding. SAA is also involved in the inflammatory ROS burst and production of inflammatory chemokines such as IL-8 (He, Sang et al. 2003) in the macrophage foam cell formation in atherogenesis (Lee, Kim et al. 2013) and in rheumatoid arthritis (Satomura, Torigoshi et al. 2013).

Aβ42 is an enzymatic cleavage fragment of the amyloid precursor protein and is the major component of senile plaques in Alzheimer's disease. Through engagement of FPR2/ALX, Aβ42 may cause neurodegeneration and pro-inflammatory responses in glial cells (Yazawa, Yu et al. 2001, Cui, Le et al. 2002). PrP106-126 causes monocyte FPR2/ALX internalization and release of pro-inflammatory mediators (Miele, Cordella-Miele et al. 1988).

1.3.4 The role of FPR2/ALX in AMI

Increasing evidence suggests that the FPR family, and their agonists, might have an important functions in the pathophysiology of I/R injury (Gavins 2010). In particular, studies showed that AnxA1 (D'Amico, Di Filippo et al. 2000) and AnxA1-derived peptides - including short sequences derived from the N-terminal region, termed peptide Ac2-12 and Ac2-26 (La, D'Amico et al. 2001, Qin, Buxton et al. 2012, Qin, Buxton et al. 2013) - are able to exert protective effects in an experimental model of myocardial ischemia-reperfusion. Ac2-26 reduced myeloperoxidase activity and IL-1β levels in infarcted murine hearts (La, D'Amico et al. 2001). Boc-2, a pan-FPRs antagonist (able to antagonize FPR1 and FPR2/ALX in human cells, and Fpr1, Fpr2 and Fpr3 in the mouse) reversed the cardioprotective actions of AnxA1 and its N-terminal peptides (La, D'Amico et al. 2001), demonstrating the involvement of the FPRs receptors in the cardioprotective properties of these molecules.

A recent publication (Qin, Buxton et al. 2013) showed that Ac2-26 is able to improve recovery of left ventricle function through activation of the FPR1 receptor. The pan-antagonist Boc-2 and the FPR1-selective antagonist CysH blocked this effect. However other insights were obtained with the use of Fpr1 null mice (Gao, Lee et al. 1999). In this study peptide Ac2-26 retained its cardioprotective properties in this transgenic colony (Gavins, Kamal et al. 2005) providing evidence for the involvement of other BOC-2 sensitive receptors. Real time PCR revealed expression of Fpr2 in the infarcted heart (Gavins, Kamal et al. 2005). Congruent with this hypothesis is the observation that FPR2/ALX ligands like LXA_4 (Chen, Wu

et al. 2013) and the Compugen peptide CGEN-855A (Gavins, Yona et al. 2003), and AnxA1 derived peptide CR-AnxA1(2-50) (Dalli, Consalvo et al. 2013) afford protective effects in rat and murine myocardial infarct models. Whilst these studies indicate the *protective nature* of FPR2/ALX in AMI, they do not shed light on the site of action and/or expression, that is, whether the leukocyte or the cardiomyocyte are the main cellular target bearing this receptor. There is evidence that peptides derived from the AnxA1 N-terminal can directly affect cardiomyocyte and exhibit protective effects against endotoxin-induced cardiac depression (Ritchie, Sun et al. 1999, Ritchie, Sun et al. 2003). Also, FPR2 is expressed in human coronary artery endothelial cells (HCAEC) where it can bind SAA inducing a pro-inflammatory phenotype like phosphorylation of ERK1/2, NF-κB and JNK, as well as release of IL-6, IL-8, G-CSF, GM-CSF, ICAM-1 and VCAM-1 (Lakota, Mrak-Poljsak et al. 2013).

Human AMI and other surgically based clamping procedures are also well known to cause aberrant PMN activation and infiltration (Goldman, Welbourn et al. 1992, Chiang, Gronert et al. 1999), giving rise to second-organ injury and subsequently longer hospitalization (Gelman 1995). Also, the delayed fall in flow in the ischemic areas - the “no reflow phenomenon” - has been directly linked to neutrophil localization (Frangogiannis, Smith et al. 2002). LXA_4 is able to modulate this PMN response (Serhan 1997, Chiang, Gronert et al. 1999) and its formation within ischemic tissue, and elevation by reperfusion (Chiang, Gronert et al. 1999), might represent an endogenous compensatory and protective role to limit PMN trafficking and PMN-mediated damage. This is not unique to LXA_4 as other FPR2/ALX agonists, like the synthetic analogue 15-epi-lipoxin (Leonard, Hannan et al. 2002,

Bannenberg, Moussignac et al. 2004), have a protective role in I/R-induced second-organ kidney and lung injury. AnxA1, for example, can reduce tissue-damage associated with ischemia-reperfusion as shown in the mesentery (Gavins, Yona et al. 2003), brain (Gavins, Dalli et al. 2007) and kidney (Facio, Sena et al. 2011). However, its potential effect on distant organ injury has yet to be determined. What is interesting, though, is the description of a non-genomic relationship between LXA_4 and AnxA1, with the short-lived lipid being able to rapidly mobilize the protein in human and mouse PMN, the end-point being reduced activation and trafficking (Brancaleone, Dalli et al. 2011).

1.4 HYPOTHESIS & AIMS

Taken together these observations lead us to propose that *FPR2/ALX (or Fpr2/3 in the mouse) and its agonists may represent a novel therapeutic target to prevent damage caused by AMI in the heart and secondary injury organs.*

For this reason the aims of this studentship are to:

1. *Understand if the cardioprotective property of AnxA1 is Fpr2/3 mediated.*
2. *Study role and modulation of Fpr2/3 in myocardial infarct, focusing on the heart.*
3. *Study role and modulation of Fpr2/3 in secondary organ damage following myocardial infarct, focusing on lungs and kidneys.*
4. *Study the effects of Fpr2/3 pro-resolving agonists in myocardial infarct.*
5. *Understand the distinct domains required by pro and anti Fpr2/3 agonists to induce downstream associated signalling.*

Understanding the potential biological functions of *Fpr2/3* in myocardial infarct, and how it could be modulated during on-going local and systemic inflammation, may represent a key step towards unveiling its biology and potentially exploiting it for drug discovery programs for novel AMI treatments. The study of the domains required only by pro-resolving Fpr2/3 agonists for the activation of the receptor help to synthetises selective and specific anti inflammatory molecules.

We postulate that exploitation of targets activated by endogenous anti-inflammatory mediators could lead to molecules effective for I/R diseases burdened by fewer side effects as their effect would be similar to mechanisms used by the body to sequester inflammation.

1.5 PUBLICATIONS

Related to this thesis

1. **Bena S**, Brancaleone V, Wang JM, Perretti M, Flower RJ. Annexin A1 interaction with the FPR2/ALX receptor: identification of distinct domains and downstream associated signaling. J Biol Chem. 2012 Jul 13;287(29):24690-7.

2. **Bena S**, Gobbetti T, Cenac N, Le Fouder P, Moyes A, Hobbs A, Vergnolle N, D'Amico M, Perretti M. A non-redundant role of the FPR2 receptor in acute myocardial infarct with the involvement of Annexin A1. *In preparation.*

Contribution to other publications during this PhD

1. Locatelli I, Sutti S, Jindal A, Vacchiano M, Bozzola C, Reutelingsperger C, Kusters D, Bena S, Parola M, Paternostro C, Bugianesi E, McArthur S, Albano E, Perretti M. "Endogenous annexin A1 IS a novel protective determinant in nonalcoholic steatohepatitis (NASH)". *Hepatology*. 2014 April.

2. Cash JL, Bena S, Headland SE, McArthur S, Brancaleone V, Perretti M. Chemerin15 inhibits neutrophil-mediated vascular inflammation and myocardial ischemia-reperfusion injury through ChemR23. *EMBO Rep*. 2013 Sep 3.

3. Nadkarni S, Cooper D, Brancaleone V, Bena S, Perretti M. Activation of the annexin A1 pathway underlies the protective effects exerted by estrogen in polymorphonuclear leukocytes. *Arterioscler Thromb Vasc Biol*. 2011 Nov;31(11):2749-59.

4. Brancaleone V, Dalli J, Bena S, Flower RJ, Cirino G, Perretti M. Evidence for an anti-inflammatory loop centered on polymorphonuclear leukocyte formyl peptide receptor 2/lipoxin A4 receptor and operative in the inflamed microvasculature. *J Immunol.* 2011 Apr 15;186(8):4905-14.

CHAPTER 2: MATERIAL AND METHODS

2.1 Chemical and reagents

Human recombinant AnxA1 was produced as published previously (Pederzoli-Ribeil, Maione et al. 2010). SAA was purchased from PeproTech Laboratories (London, UK), and C43 was a generous gift from Amgen (Thousands Oaks, CA). *N*-tert-butoxycarbonyl-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe (Boc2) was bought from Tocris (UK). The JAK/STAT inhibitor Tyrphostin AG 490 was obtained from Sigma-Aldrich (USA). The MEK1 inhibitor PD98059 and the calcium inhibitor BAPTA-AM were obtained from Cell Signaling Technologies (Hertfordshire, UK) and Calbiochem, respectively. F1, B11 and GB4 primers were synthesized by Thermo Electron, MA.

2.2 Fpr2/3 KO colony

Male Fpr2/3 KO mice and wild type (WT) littermate controls (6-7 weeks old) were maintained on a standard chow pellet diet and had free access to water, with 12 hours light dark cycle. Animal work was performed in accordance with the U.K. Home Office Animals (Scientific Procedure) Act 1986.

Our Fpr2/3 KO mice are generated by the deletion of both the murine Fpr2 and Fpr3 genes (Figure 3). Of interest, the Fpr1 gene is transcribed from a different strand to Fpr2 and Fpr3 and was not modified.

The transcription of the Fpr2 and Fpr3 genes was prevented with the insertion of a GFP PGK-Neo cassette (Figure 7) creating a transgenic colony characterised by an in-frame green fluorescence protein (GFP) gene ‘knocked-in’ (Dufton, Hannon et al. 2010). The PGK-Neo is a vector that contains the phosphoglycerate kinase I

(PGK) promoter and a neomycin resistance gene (neo). Using the PGK-Neo recombinase-mediated cassette exchange the genetic modification is permitted by by exchange of a pre-existing gene cassette with an analogous cassette carrying the "gene of interest" (GFP).

The GFP is a 238 amino acid protein with a MW of 26.9kDa. It is able to exhibit bright green fluorescence when exposed to blue light (Tsien 1998). The GFP from the jellyfish *Aequorea victoria* has a major excitation peak at the wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 509 nm, which is in the lower green portion of the visible spectrum (Prendergast and Mann 1978). In cell and molecular biology, the GFP gene is a marker used for example to monitor physiological processes or visualize protein localization.

The genotype of the Fpr2/3 KO mice was confirmed by PCR screening of ear clip DNA using the Extract-N-Amp Tissues kit (Sigma-Aldrich, XNAT2-1KT). The primers used are F1 (Fpr2 forward – TGAGTGTCATGTCAGAAGGAGCC), B11 (Fpr2 reverse for WT – CGGAATCCAGCTACCCAAATC) and GB4 (Fpr2 reverse for KO –ATAACCTTCGGGCATGGCACT). The F1/B11 pair produces a band of 233 bp from the wild type allele, whereas F1 and GB4 produce a band of 351 bp for the allele of Fpr2/3 KO mice. Cycling condition were 94°C for 5min, 35 cycles of 94°C for 30min, 55°C for 30min, 72°C for 15min and 72°C for 7min.

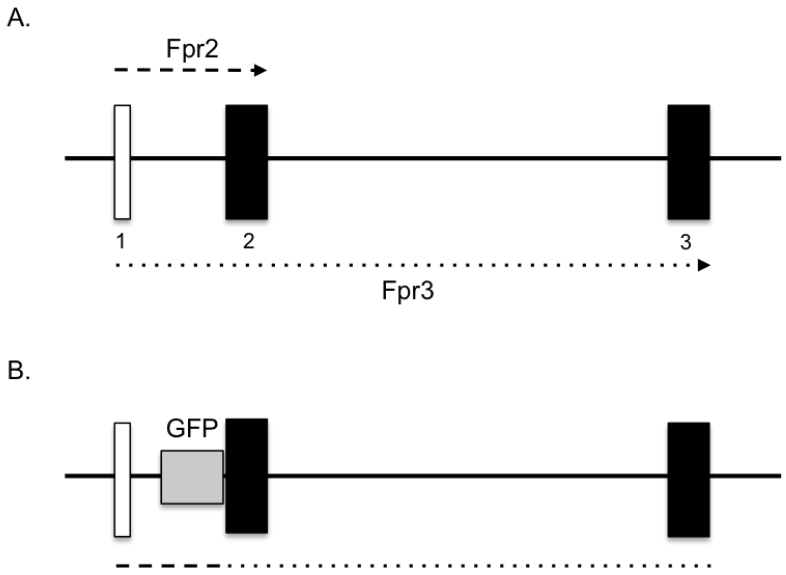


Figure 7: Schematic representation of the Fpr2/3 GFP KO mice genome.
The construct of our transgenic Fpr2/3 KO mice were characterised by deletion of the Fpr2 and Fpr3 gene and the insertion. (A) The Fpr2 gene was transcribed from exon 1 (not coding exons) and exon 2 (coding exon). Fpr3 was transcribed by exon 1 and exon 3 (coding exon). Exon 2 was excluded. (B) The GFP PGK-Neo cassette was inserted in reverse orientation into intron 1 of Fpr2 and fused in-frame (vector shown in grey) with the ATG start codon.

2.3 Quantification of myocardial tissue injury

Unlike after permanent occlusion of a coronary artery where there is necrosis of the entire coronary territory, infarctions with ischemia and reperfusion of the heart is variable. Therefore there is a need to clearly demarcate the area perfused by the occluded artery and quantify the infarct size in that area. For this reason at the end of the 2 hours of reperfusion period the LADCA was re-occluded and 100 µl of Evans Blue dye (1% w/v) (Sigma-Aldrich, USA) was injected into the left ventricle to distinguish between perfused and non-perfused areas of the heart, the Area At Risk (AAR). The Evans Blue dye stains the perfused myocardium, while the occluded vascular bed remains uncoloured. Next the mice were killed and the heart excised and excess dye was washed with saline.

After the removal of the atria and the right ventricular wall the AAR (non perfused – pink) was separated from the rest of the left ventricle (perfused – blue). Both tissues were weighed and the AAR was expressed as a percentage of the left ventricle. The AAR was then cut into small pieces and incubated with the reducing agent p-nitroblue tetrazolium (NBT, 0.5 mg/ml) (Sigma-Aldrich, USA) for 10 minutes at 37°C. This technique relies on the ability of dehydrogenase enzymes and cofactors in health tissue to react with tetrazolium salts to form a dark blue color. Infarcted tissue lack in dehydrogenase activity and fail to stain. Not stained pieces were weighted to determine the infarct size expressed as percentage of the weight of the area at risk.

2.4 Histology

2.4.1 Tissues collection and paraffin embedding process

After collection, tissues were washed in PBS and fixed with 10% formalin (buffered with 0.1M NaH_2PO_4 and 0.1M Na_2HPO_4) for 24h at 4°C before the paraffin embedding procedure was processed. Embedded tissues were then cut in serial paraffin sections (5µm) using a standard microtome. Sections were then mounted on slides, dried overnight in an oven (45°C) and stored at room temperature until required. All sections were used for the experiments within 2 days from cutting.

2.4.2 Hematoxylin/Eosin staining – GFP

Formalin fixed paraffin embedded tissue sections were deparaffinized through two seven-minutes changes in Histoclear™ and rehydrated with 100% ethanol for two washes of five minutes followed by five minutes in distilled water. Next the tissues were immersed for 10-30 seconds in Haematoxylin, washed with running tap water. Tissues were then immersed in 1% Acid/Alcohol (70% EtOH, 1% HCl, 29% dH_2O) for 10 seconds and washed and differentiated under running tap water. Afterwards tissues were immersed in eosin for 5 minutes, washed in tap water, and dehydrated by treatment for 5 minutes with 70% ethanol, 90% ethanol, 100% ethanol and twice with Histoclear™. Lastly the slides were covered with a coverslip and mounted with DPX.

To allow GFP detection the tissues were exposed to blue light for the intensity indicated. ImageJ software was used to quantify the fluorescence intensity.

2.4.3 Immunohistochemistry – Immunoenzyme method (HRP)

Formalin fixed paraffin embedded tissue sections were deparaffinized and rehydrated with Histoclear and ethanol as described before. Once hydrated, sections were rinsed with dH_2O followed by 5 minutes of TBS 1X (Tris HCl, Tris Base, NaCl). To counteract the antigen masking effects of formalin fixation, epitope retrieval was performed by heating the tissue sections for 20 minutes at 95°C followed by 5 minutes at room temperature in pre-warmed Buffer Retriever (Dako, UK) using as a source of heat a hot water bath. After antigen retrieval the sections were treated with Peroxidase blocking (Dako, UK) followed by Avidin and Biotin (Vector, UK) for 10 minutes each to block the endogenous corresponding biotin that may result in high and non-specific background staining. The sections were then washed for two times for two minutes with Tris-Buffered Saline (TBS) and incubated for 10 min. at room temperature with Protein Block Serum Free (Dako) to reduce non-specific staining blocking.

Subsequently the tissues were incubated with the primary antibody (Anti rabbit polyclonal Fpr2 – Santa Cruz) for 1 hour in humidified chambers. Next the sections were washed three times in TBS with 5 minutes between each wash and incubated with the biotinylated secondary Ab (1:200, Swine anti-rabbit - Dako) for 1 hour at room temperature. The secondary antibody was rinsed off by three 5-minutes washes in TBS and sections were further incubated with a preformed Avidin and Biotinylated horseradish peroxidase Complex (ABC Kit – Vector, UK) for 30 minutes. The ABC substrate solution was prepared using 1µl of Avidin and 1µl of the Biotinylated enzyme in 1 ml of TBS and allowed to react 30 minutes at room temperature before use. Sections were then washed three times in TBS (5 minutes

per wash) and developed using the Liquid DAB+Substrate Chromogen System (Dako, UK). The substrate working solution was prepared adding 1 drop (20µl) of the DAB Chromagen per mL of Sustrate Buffer. This substrate-chromagen system is a system suitable for peroxydase-based immunohistochemical (IHC) staining methods. Upon oxidation, DAB forms a brown end-product at the site of the target antigen. The development reaction was stopped in distilled water. Sections were then counterstained with hematoxylin for 30 seconds and hydrated through graded ethanol solutions (70%, 90%, 100%, 5 minutes each) and Histoclear™ twice, for 5 min each. To finish the sections were mounted using a coverslip and a mounting DPX.

Primary and secondary antibodies were diluted in ready-to-use Antibody diluent with Background Reducing Components (Dako, UK). Negative staining control experiments were performed omitting the primary antibody.

2.5 Cell culture

Stably transfected FPR1 and FPR2/ALX HEK-293 cells (Human Embryonic Kidney cells) were available in the laboratory – and been described by (Hayhoe, Kamal et al. 2006); cells were maintained in culture with DMEM F-12 supplemented with 10% FCS, gentamicin (50 µg/ml), Non-essential Amino Acids (500 µg/ml) and geneticin (200 µg/ml) solution. The HEK-293 cells transfected with the chimaeric FPR1/FPR2 receptors were obtained with distinct domains of the FPR1 receptor replaced with FPR2/LAX amino acid sequences (Le, Ye et al. 2005). These cells

were cultured in DMEM with 10% FCS, L-glutamine (500 µg/ml), Penicillin-Streptomycin mix (500 µg/ml), Hepes 1M and geneticin solution (800 µg/ml).

2.6 Cells and tissues lysis and Protein Assay

Protein content (aliquots diluted 1:100) was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) that measure the total protein concentration compared to a protein standard (Figure 8). The assay combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. Briefly, the first step is characterized by the chelation of copper with protein in an alkaline environment (with sodium potassium tartrate) to form a light blue complex. Subsequently, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed resulting in an intense purple-colored reaction product. The BCA/copper complex exhibits a linear absorbance at 562 nm with increasing protein concentrations.

Cell and tissue lysis. Cells and tissues were lysed in lysis buffer containing Tris HCl, 1% Triton and the protease inhibitors aprotinin (1mM), Leupeptin (10µM), PMSF (200µM), β-GP (1mM) and DTT (1mM) and phosphatase inhibitors NaF (1mM), Na_3VO_4, (1mM). Tissues were treated with lysis buffer (100µl/10µg tissue) in round-bottom microfuge tubes and subjected to centrifugation in the homogenizer (Precellys 24 – Stretton Scientific LTD, UK) at 5,000 rpm for 50 seconds twice, followed by a centrifugation at 10,000 rpm for 10 minutes. Cells were resuspended in 100µl of the lysis buffer followed by a mechanical lysis by syringe.

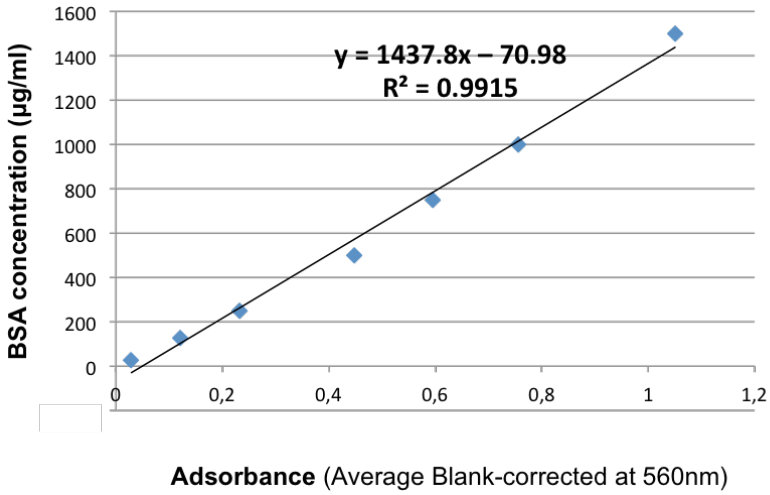


Figure 8: Standard curve of bovine serum albumin (BSA).

A series of dilutions of known concentration of BSA were prepared and assayed alongside the unknown protein. A standard curve of BSA was prepared by plotting the measurement of BSA standard *vs*. its concentration. The concentration of each unknown protein was then determined and reported with reference to the standard curve of the BSA. Each sample was analysed in triplicate and the average was blank-corrected. Equation and R^2 value (Pearson Coefficient of Determination) are shown.

2.7 Enzyme-linked immunosorbent (ELISA) assay

ELISA uses a solid-phase enzyme immunoassay (EIA) to detect the presence of an antigen in a sample. There are different types of ELISA. Here the "Sandwich" ELISA was used. Briefly, a 96 wells plate was coated with a known quantity of capture antibody. Subsequently any nonspecific binding sites on the well were blocked and the samples and the standards were added to the plate allowing any antigen present in the sample to bind to the capture antibody. The plate was then washed to remove unbound antigen and a detecting antibody, whose bind to the antigen, was added. Enzyme-linked secondary antibodies were then applied to bind to the detecting antibody. The detection antibody was conjugated with an avidin-horse radish peroxidase (HRP) and visualized with a 3,3',5,5'-tetramethylbenzidine (TMB), an HRP substrate solution, resulting in the development of blue. The colour development was stopped by addition of 2N H_2SO_4 changing to yellow. The absorbance of the plate wells was measured at 450nm to determine the presence and quantity of antigen. The sample values were read off the standard curve (Figure 5).

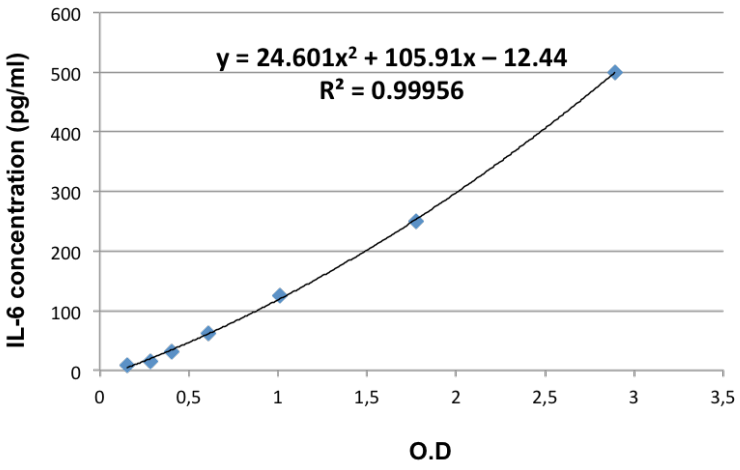
IL-6, TNFα, IL-1β, IL-10 and CCL2 were measured by murine Ready-Set-Go ELISA kit according to manufactures instructions (eBioscience). Tissues were diluited 1:20 and plasma 1:2 (Figure 9)

Keratinocyte-derived Cytokine (KC) ELISA. Mouse KC levels in plasma (1:2 dilution) and tissues (1:20 dilution) were measured using the Mouse CXCL1/KC Elisa Kit (R&D System).

Mouse Serum Amyloid A (SAA) ELISA. Levels of SAA were measured using a mouse SAA Elisa kit (immunological Consultants Laboratory, Newburg, OR). Samples were diluted 1:20 for tissues and 1:2 for plasma.

Regulated upon activation, Normal T cell expressed and presumably secreted (RANTES) ELISA. Mouse RANTES levels were measured in tissues (1:20 dilution) using the Mouse RANTES Instant ELISA (eBioscience).

Troponin I ELISA. Troponin-I levels were measured using a mouse cardiac troponin-1 elisa kit (Life Diagnostics). The high sensitivity cTnI Elisa recognizes an epitope on mouse cTnI that is resistant to preoteolysis in mouse plasma. Plasma samples were diluted four-fold (Figure 9).



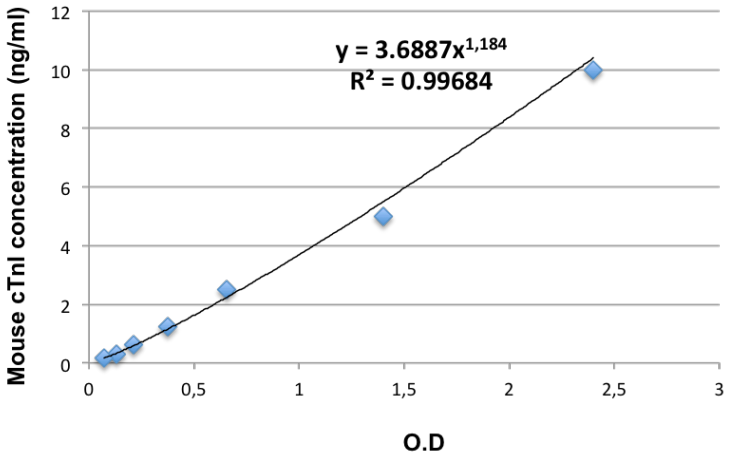


Figure 9: Standard curve of IL-6 and mouse cTnI Elisa kit

Examples of standard curve made with eBioscience Ready-Set-Go (IL-6) and Life Diagnostics (mouse cTnI) ELISA kit. Standards were prepared in serial dilution and assessed, in duplicate, together with the samples according to manufacturing instructions. Equation and R^2 value (Pearson Coefficient of Determination) are shown.

2.8 Measurement of myeloperoxidase (MPO) activity

MPO enzyme activity, index of granulocyte infiltration, was assayed by measuring hydrogen peroxide dependent oxidation of 3,3',5,5'-tetramethyl-benzidine (TMB). Blood-free tissue samples were homogenised in cell lysis tubes (Peqlab, UK) containing a solution of 0.5% hexadecyltrimethylammonium bromide (HTAB) dissolved in phosphate buffer solution (pH 6.0). The homogenized tissues were centrifuged at 15000rpm for 5 min. Supernatants were added to a buffer containing distilled water, 1% hydrogen peroxide, and *O*-dianisidine dihydrocholoride solution. Optical density readings were taken for 1 min at 30 s intervals at 450 nm.

2.9 Western Blot analysis

HEK-293 transfected with FPR1, FPR2/ALX and the chimeric receptors, HEK-293-GFP cell lysates, murine bone marrow cells, bone marrow derived macrophages and mouse tissue lysates were analyzed using the SDS-PAGE technique. Samples were boiled in 6x Laemmli buffer for 5 min, and analyzed by standard SDS-polyacrylamide gel electrophoresis (10% or 12% acrylamide gels) for 90 minutes at 110V. The separated proteins were then electrophoretically transferred to polyvinylidene difluoride membranes at 4°C for 75 minutes using 100V.

Membranes with AMI samples were then incubated with the mouse Phospho-Stat3 (Tyr705) (1:1000, Cell Signalling, MA) and the Stat3 (79D7) antibodies (1:2000, Cell Signalling, MA). All the antibodies were diluted in Tris-buffer saline solution containing 0.1% Tween 20 (TBST) and 1% non-fat dry milk or TBST with 5% BSA

(phosphorylated proteins) overnight at 4°C. Membranes were then washed for 30 min with TBST with the solution being changed at 10-min intervals and incubated with the secondary antibody (HRP-conjugated goat anti-rabbit 1:2000, Dako, Cambridge, UK), for 1h and 30 minutes at room temperature.

HEK-293 FPR1, FPR2/ALX and cell clones were incubated with rabbit anti-phospho-p44/42 MAP kinase (1:1000 dilution) and anti-total-p44/42, clone 137F5, (Cell Signalling, Cambridge, USA, 1:1000 dilution) diluted in Tris-buffer saline solution containing 0.1% Tween 20 (TBST) and 1% BSA overnight at 4°C. Membranes were washed for 30 min with TBST and incubated with secondary antibody (HRP-conjugated goat anti-rabbit 1:2000, Dako, Cambridge, UK), for 2 h at room temperature. After stripping, membranes were also incubated with anti-β-actin (1:10,000; SigmaAldrich) in TBST and 5% non-fat dry milk. Human actin was detected with HRP-conjugated goat anti-mouse (1:5000).

In all cases proteins were then detected using enhanced chemiluminescence (ECL) detection kit and visualized on Hyperfilm (GE Healthcare, Little Chalfont, UK).

2.10 Caspase 3 activity assay

The activity of caspase 3 was analyzed using the Caspase 3 Assay Colorimetric Kit (Abcam). Caspase 3 cleaves the Poly(ADP-ribose)polymerase and its derived tetrapeptide DEVD during cells death by apoptosis. Derivatives of DEVD conjugated with a chromophore used as substrate for enzyme activity assay of

caspase 3. This assay is based on spectrophotometric detection of the chromophore p-nitroaniline (p-NA) after cleavage from the labeled substrate DEVD-p-NA. The p-NA light emission is quantified at 405nm.

2.11 Quantitative Real Time PCR

The assay was processed as previously shown (Renshaw, Montero-Melendez et al. 2010). Briefly, RNA was extracted using an RNeasy Kit (Qiagen, 74104) according to the manufacturer's instructions. Total RNA was normalized at 5ug/reaction. cDNA was produced using SuperScript III (Invitrogen Ltd, Paisley, UK). Samples were normalized to 100ng of cDNA per well and loaded in duplicate for each gene using a Power SYBR Green PCR Master Mix (AB 4367659). Specific QuantiTect primer assays (Qiagen Ltd, UK) were used. GAPDH was used as endogenous control. PCR ramping protocols were standardized for QuantiTect primer assay sets at 95°C for 15min followed by 40 cycles of 15 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C. Data were analysed using the relative quantification method (Schmittgen and Livak 2008). Following this method the response produced in control mice (NO AMI) was taken as 1 and all the data represent the upregulation (>1) or downregulation (<1) of the mRNA expression in mice after AMI.

2.12 Human polymorphonuclear leukocyte isolation

Peripheral blood was collected from healthy volunteers by intravenous withdrawal in 3.2% sodium citrate solution (1:10). All healthy volunteers gave oral and written

consent and cell separation was covered by ethical approval 05/Q0603/34 (East London and The City Research Ethics). Granulocytes (PMNs) were isolated from blood *via* density centrifugation on Histopaque 1119/1077 (Sigma-Aldrich, Poole, UK) gradient according to the manufacturer's instructions and suspended in PBS containing 0.5% BSA. Contaminating erythrocytes were removed by lysis with cold milliQ water.

2.13 Peritoneal cells isolation – Biogel method

The peritoneal cavity is a unique compartment where several immune cells reside and from where resident macrophages are commonly collected for functional studies. The cavity of Fpr2 KO and WT mice was lavaged with PBS with 10% ethylenediaminetetraacetic acid (EDTA).

Biogel method. One milliliter of sterile Biogel (2% Biogel solution in PBS) was injected in the peritoneal cavity of the mouse. After 4 days, mice were sacrificed by cervical dislocation and the cells were collected using PBS with 10% EDTA. The cells were then filtrated with 70-µm strainer and centrifuged at 1200 rpm for 10 minutes. Subsequently cells were re-suspended in RPMI medium with 10% heat-inactivated fetal calf serum (FCS) and 0.5 ng/ml gentamicin.

2.14 Culture of Bone Marrow Macrophages

2.14.1 Generation of conditioned medium

L929 fibroblasts were plated in T75 cm^2 flasks containing DMEM supplemented with 10% FCS and maintained in an incubator with 37°C, 5% CO_2 and 95% O_2. When the cells became confluent, after a wash with sterile PBS, they were detached with Trypsin and plated in T175 cm^2 flasks with 100ml of fresh medium. On day 3 of culture, the supernatant medium was collected, centrifuged, filtered through a 0.22 μm filter and used as conditioned medium for the direction of bone marrow pre-cursor cells towards a mature macrophage phenotype. The conditioned medium was made with DMEM medium containing 20% FCS, 30% L929 supernatant and 50μg/ml gentamicin.

2.14.2 Harvest of bone marrow cells

Young (4-6 week old) Fpr2/3 KO and WT male and female mice were sacrificed by cervical dislocation. To dissect away the skin and expose the hind limbs a transverse cut through the abdomen skin was performed and, in order to expose the femurs and tibia, the muscles attaching the hind limbs to the pelvis were dissected. Subsequently the femurs and the tibia were washed in a Petri dish with 70% ethanol for 1 minute and then moved to another Petri dish containing sterile PBS. After the cutting of the bones epiphyses a 25-gauge needle, connected with a 5 ml syringe containing sterile PBS, was placed into the bone shaft and the bone marrow was flushed out in a 50 ml falcon tube. The cells were then filtered through

a 70µm strainer and centrifuged for 5 minutes at 1200 rpm. All of the protocol was performed in a sterile flow cabinet.

2.14.3 Bone marrow cells differentiations

The cells, counted using a Neubauer haemocytometer, were plated at a concentration of $2x10^6$ml in 10cm sterile Petri dishes with 10ml of conditioned medium. They were incubated in a humidified incubator for 5 days to allow proliferation and differentiation. 6ml of fresh conditioned medium was added on day 3. On day 5, non-adherent cells were removed by washing with PBS and adherent cells were harvested using a cell scraper. The bone marrow which was flushed from the femurs and the tibia gave approximately 50-80 x 10^6 cells per mouse and resulted in approximately 40 x 10^6 macrophages following 5 days of differentiation. The differentiation of macrophages was confirmed by flow cytometry using the F4/80 antigen staining that revealed 83% of macrophages at day 5 (Figure 10) (Weischenfeldt and Porse 2008). F4/80 is a mature mouse cell surface glycoprotein expressed at high levels on various macrophages (Austyn and Gordon 1981).

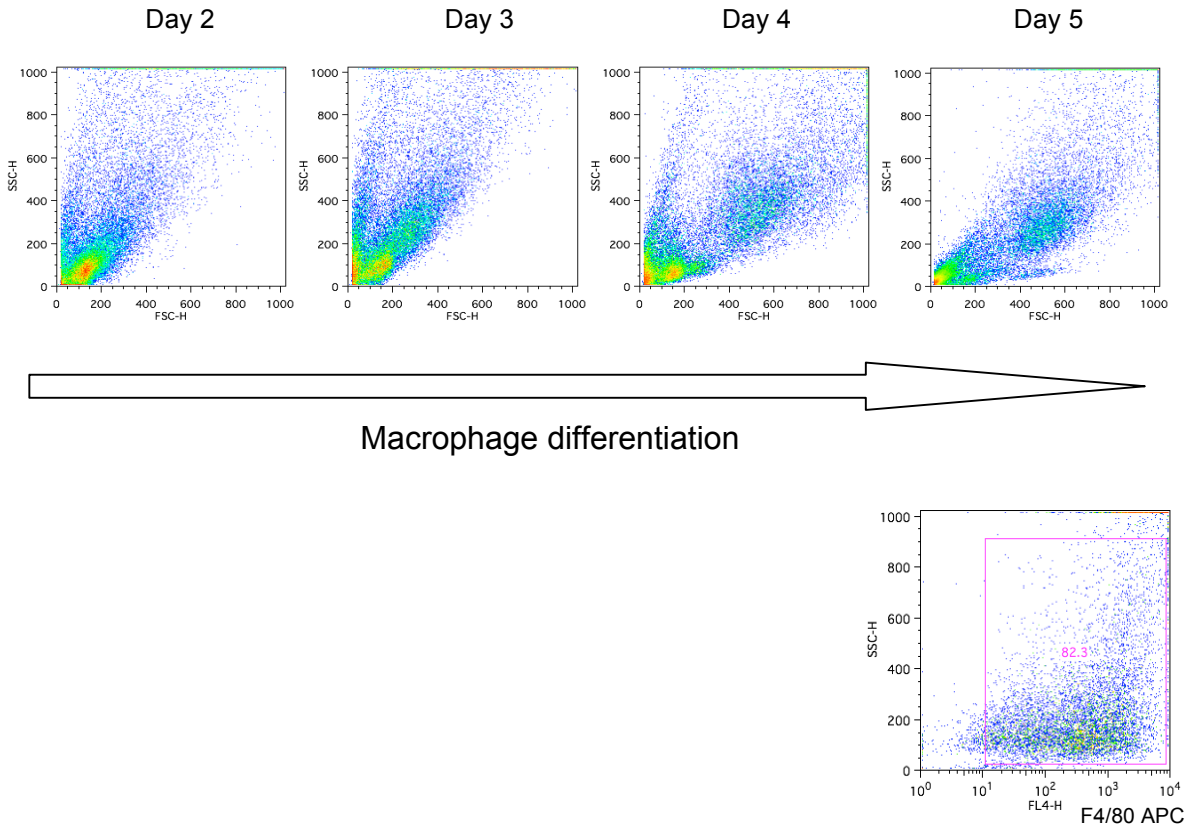


Figure 10: Differentiation of bone marrow cells in macrophages.
Bone marrow cells have been differentiated using L929 conditioned medium for 5 day. The differentiation was confirmed by flow cytometry using the F4/80 antigen staining that revealed 83% of macrophages at day. During each day of differentiation the FSC/SSC plot revealed the gradual increase of the macrophages population.

2.15 Flow cytometry analysis

2.15.1 HEK293 transfected cells

HEK293 FPR1 and FPR2/ALX cell surface expression was measured by incubation with anti-FPR1 or anti-FPR2/ALX mAb (1:100 dilution in both cases; from R&D System, Abingdon, UK and Genovac, Brussels, Belgium, respectively); a final staining with a rabbit antimouse IgG (1:200 dilution, clone STAR9B; Serotec) was then conducted. Flow cytometry analysis was performed analyzing ≥10,000 events using a FACScalibur flow cytometer (Becton Dickinson, San José, CA) equipped with CellQuest software (Becton Dickinson) and analysed using FlowJo (Version 9.2, Treestar Inc, Stanford, CA). Results are reported as median fluorescence intensity (MFI) units.

2.15.2 GFP detection

Whole blood, peritoneal cells, bone marrow cells and bone marrow derived macrophages were analyzed by flow cytometry (FACS). The FACS analysis was used to monitor the fluorescent properties of the Green Fluorescence Protein. GFP expression was measured in the FL1 channel (natural fluorescence). To amplify the signal, a rabbit polyclonal anti-GFP antibody (Invitrogen, Molecular probes) and a PE-conjugated donkey anti-rabbit IgG secondary (1:200 dilution, eBioscience) were used. When the anti GFP antibody was used a cell membrane permeabilization with Saponin was performed to allow an intracellular staining.

Extracellular staining*:* cells were resuspended in round-bottom 96-well plate (400.000 cells/well) with 100µl ice-cold PBS containing 0.2% bovin serum albumin (BSA) and 1.3mM Cacl2 (PBC). Cells were washed (400g, 30s) before non-specific binding was blocked with 16mg/ml human immunoglobulin g (IgG) for 5 min at 4°C. Cells were then washed and incubated with the primary antibody for 40 min at 4°C followed, after 3 washing, by the secondary antibody for 20 min at 4°C. Cells were then washed prior to fixation with 4% formaldehyde (PFA) (10 min; 4°C) and resuspended in 200µl PBC for analysis.

Intracellular staining: cells, incubated with 16mg/ml human immunoglobulin g (IgG) for 5 min at 4°C to block non-specific binding were then fixed with 4% PFA for 10 min at 4°C. Cells where then washed with PBC containing 0.1% saponin (From Saponaria species – Sigma) to allow permeabilization of the cell membrane. Then, the staining with the primary and the secondary antibody diluted in PBC supplemented with 0.1% saponin was performed as described for the extracellular staining. Cells were washed with PBC/0.1% saponin between each staining step. Finally cells were washed twice in PBC without saponin before being re-suspended in 200µl PBC for analysis.

Whole blood staining: blood aliquots (0.5-1 ml of mouse blood) were collected by cardiac puncture using 3.2% tri-sodium citrate, from both WT and Fpr2/3 KO mice under terminal anesthesia. Aliquots were diluted to 4ml with PBC, centrifuged at 400g for 5min and the cell pellets obtained stained, as above, in 15-ml tubes. Following staining the cell pellets were washed twice and lysed using 500µl/tube of Whole Blood Immuno-Lyse to allow the lysis of the red blood cells. Cells were then

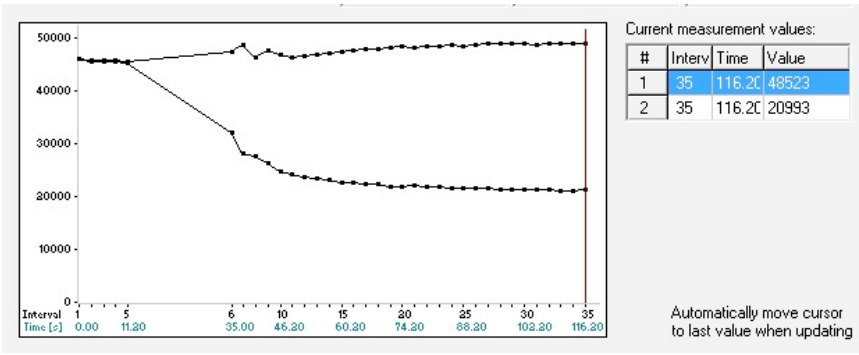
washed twice, fixed with 2% PFA and resuspended in 250µl PBC for analysis. To monitor the nature of the cells an extracellular staining with APC conjugated Ly6G (GR-1, Granulocytes) and APC or PE conjugated F4/80 (Monocytes/Macrophages) was also performed.

Flow cytometry analysis was performed analyzing ≥10,000 events using a FACScalibur flow cytometer (Becton Dickinson, San José, CA), CellQuest software (Becton Dickinson) and analysed using FlowJo analysis software (Version 9.2, Treestar Inc, Stanford, CA). Results are reported as % of positive cells and/or as median fluorescence intensity (MFI) units for specific fluorescence.

2.16 Calcium mobilization assay

HEK-293 cells or freshly prepared PMNs were incubated with 2µM Fura 2-AM (Molecular Probes, Invitrogen, Paisley, UK) and 1µM Pluronic Acid F127 (Molecular Probes, Invitrogen, Paisley, UK) in extracellular solution (13mM glucose, 10mM HEPES, 147mM NaCl, 2mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$ at pH 7.3) at 37°C for 1 h in the dark. Subsequently, cells were washed x3 with the extracellular solution, added to 96-well plates prior to stimulation with agonists at indicated concentrations. Ionomycin (1µM), a Ca^{2+} ionophore, was used as a positive control. Mobilization of intracellular calcium was measured by recording the ratio of fluorescence emission at 510 nm after sequential excitation at 340 nm and 380nm using the NOVOstar microplate reader (BMG Labtech, Aylesbury, UK) (Figure 11).

A.



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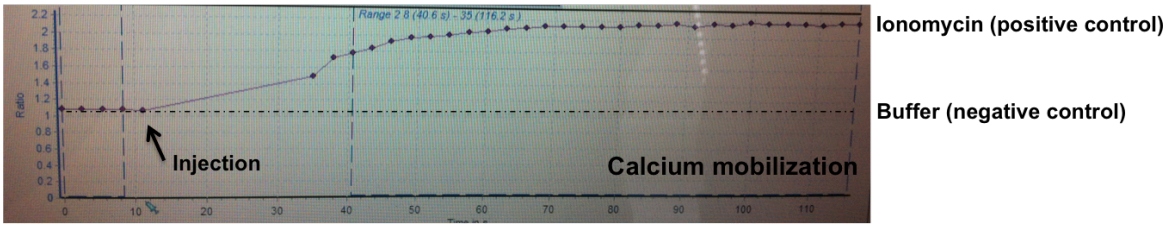


Figure 11: Calcium mobilization induced by Ionomycin in HEK-293 cells

Fura-2AM loaded HEK-293 cells were stimulated with ionomycin and calcium flux was recorded using the NOVOstar microplate reader (BMG Labtech, Aylesbury, UK). (A) Fura-2, a cell-permeable acetoxymethyl (AM) ester form of Fura-2, is excited at 340 nm and 380 nm of light. (B) The ratio of the emissions at those wavelengths is directly correlated to the amount calcium mobilization induced by Ionomycin. Buffer was used as negative control.

2.17 Liquid chromatography/tandem mass spectrometry measurement

The liquid chromatography mass spectrometry assay has been performed by the laboratory of Prof. Natalie Vergnolle in Tolouse (France) using plasma samples rapidly frozen in liquid nitrogen and stored at -70 degrees.

2.17.1 Lipid extraction

Plasma samples were submitted to solid-phase extraction using HRX-50 mg 96-well (Macherey Nagel, Hoerd, France). Briefly, the plate was conditioned by successive washing with MeOH (2 mL) and H_2O/MeOH (90:10, 2 mL). The sample was loaded at flow rate of 0.1 mL/min. After complete loading, the plate was washed with H_2O/MeOH (90:10, 2 mL). After drying under aspiration, lipid mediators were eluted with MeOH (2 mL). Solvent was evaporated under nitrogen and samples were dissolved with MeOH and stored at -80 °C for Liquid chromatography/tandem mass spectrometry measurements.

2.17.2 Measurement

6kPGF_1a, TXB_2, PGE_2, PGA_1, 8-isoPGA_2, PGE_3, LxA_4, LxB_4, RvD1, RvD2, 7-MaR1, LTB_4, LTB_5, Pdx, 18-HEPE, 5,6-DiHETE, 15-HETE, 12-HETE, 8-HETE, 5-HETE, 17-HDoHE, 14-HDoHE, 14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET and 5-oxo-ETE were quantified in plasma samples. To simultaneously separate 27 lipids of interest and 3 deuterated internal standards (LxA_4-d5, LtB_4-d4, 5-HETE-d8) LC-MS/MS analysis was performed on UHPLC system (Agilent LC1290 Infinity) coupled to

Agilent 6460 triple quadrupole MS (Agilent Technologies) equipped with electro-spray ionization operating in negative mode. Reverse-phase UHPLC was performed using ZorBAX SB-C18 column (2.1 mm, 50 mm, 1.8 µm) (Agilent Technologies) with a gradient elution. Mobile phase A consisted of water, ACN and FA (75:25:0.1;v/v/v); Solvent B: ACN, FA (100:0.1, v/v). Compounds were separated with a linear gradient to 85 % B in 8.5 min and then to 100 % B in one more minute. Isocratic elution continued for 1 min at 100 % B then 100 % A was reached in 1 min and maintained for 1 min. The flow rate was 0.35 mL/min. The autosampler was set at 5 °C and the injection volume was 5 µL. Data were acquired in Multiple Reaction Monitoring (MRM) mode with optimized conditions (fragmentors and collision energy). Peak detection, integration and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies). Mass Hunter quantitative (MH Quant) analysis software was used to generate calibration lines. For each standard, the validity of the calibration line was evaluated by comparing the deviation between back-calculated amounts and nominal amounts of standards. For the calibration curve, a linear regression with a weight factor of 1/X was applied for each compound. By the use of this factor, calibration line with a coefficient of correlation over 0.998 and an accuracy ranging from 85 % to 115 % was obtained. The limit of detection (LOD) and the limit of quantification (LOQ) were determined for the 13 compounds using signal to noise ratio (S/N). All values under the LOQ were not considered. Importantly, blank samples were evaluated, and their injection showed no interference (no peak detected), during the analysis

2.18 Assessment of cardiac function

Basal cardiac function of WT and Fpr2/3 KO mice in normal condition was measured by Ken Suzuki laboratory at the William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary, University of London, London, United Kingdom. Briefly, cardiac function of WT and Fpr2/3 K0 mice was measured by using echocardiography (Vevo−770, VisualSonics) as previously described (Fukushima, Varela-Carver et al. 2007). Diastolic and systolic LV endocardial areas at the papillary muscle level were measured from parasternal short-axis views, from which LV fractional area change (LVFAC) was calculated. Hemodynamics parameters were measured by catheterization (SRP−320/PVAN3.2, Millar Instruments and Chart 5 software, ADInstruments) as described previously (Narita, Shintani et al. 2013).

2.19 Statistics

Experiments were performed with group of at least 5 mice and results expressed as mean ± SEM (Standard error of the mean). Statistical differences between 2 groups were determined with the unpaired Student *t* test; more than 2 groups were analysed using one- or two-way analysis of variance (ANOVA) upon satisfaction of homogeneity of variances. Intergroup analyses were assessed with the Dunnett or Bonferroni tests, as appropriate.

The unpaired Student *t* test compares one variable: the means of two groups. We used the unpaired test because the samples were collected from two different

populations or from randomly selected individuals from the same population at different times. The test follows a Gaussian distribution; a theory that tells the probability that an observation would fall between any two real numbers. The null hypothesis is that the means of the samples are equal for the two groups.

In an experiment with more than two groups, a multiple two-sample t-tests would increase the chance to have a type I error: a rejection of the null hypothesis. For this reason to compare the data of two or more group the analysis of variance was used.
The ANOVA tests the null hypothesis that samples in two or more groups have the same mean values using the *F*-distribution, an asymmetric distribution characterised only by a minimum value of 0. Samples must be normally distributed. The one-way ANOVA analyses the effect of one independent variable, the two-way ANOVA the effect of more than one independent variable and multiple observations for each independent variable. Indeed, the two-way ANOVA measures the effect of contributions of each independent variable and also if there is an interaction effect between the independent variables.

One- or two-way ANOVA test were followed by Dunnett or Bonferroni post tests. The Dunnett test compares each treatment with a control value. The Bonferroni test compare selected pairs of means.

In all the experiment, a $p<0.05$, described the p value as the probability of getting the results given that the null hypothesis is true, was taken as a threshold for rejecting the null hypothesis. All analyses were performed using GraphPad Prism 5.0 software.

CHAPTER 3: VALIDATION OF FPR2/3 KO MICE AND GFP EXPRESSION

3.1 Rationale

The FPR2/ALX pathway is part of an endogenous counter-regulatory circuit crucial for the resolution phase of the inflammatory response. Whereas the levels of its agonists are often quantified in several experimental and clinical inflammatory states, the potential modulation of specific receptors is almost over-looked and partially unexplored. Related to the FPR2/ALX pathway, AnxA1 gene promoter activity was studied in a spatial and temporal fashion during experimental inflammatory and septic settings (Damazo, Yona et al. 2006). Another link between these pathways is represented by the fact that expression of FPR2/ALX can be induced by glucocorticoids in leukocytes (Sawmynaden and Perretti 2006) and in experimental dermatitis (Hashimoto, Murakami et al. 2007).

We propose that receptor modulation could also occur in disease and/or after therapeutic treatment.

To understand how FPR2/ALX is involved in the inflammatory response we thought it was important to investigate how its expression could be regulated in vivo. For this reason we have generated a novel Fpr2/3 null mouse which bears an in-frame green fluorescence protein (GFP) gene 'knocked-in' (Dufton, Hannon et al. 2010) and we developed novel analytical protocols to monitor GFP expression as a reporter for Fpr2/3 gene promoter activity. Specifically, we used biochemical and imaging protocols aimed at determining cell-associated GFP expression in cells and tissues harvested from the Fpr2/3 null colony.

These techniques helped to understand if and how inflammation could modulate FPR2/ALX expression and this achievement would provide important knowledge on the biology of this receptor and on the opportunity that selective agonists may be relevant as novel anti-inflammatory therapeutics.

3.2 Genotyping of Fpr2/3 KO mice

The genotype of Fpr2/3 KO mice, on a C57BL/6 WT background, was confirmed. Mice were screened by PCR of ear clip DNA for the transmission of the Fpr2/3 allele (Figure 12). Using our strategy the deletion of the Fpr2 gene also deleted the Fpr3 gene since it incorporates exon 1 of Fpr2.

The primers used were F1 (Fpr2 forward – TGAGTGTCATGTCAGAAGGAGCC), B11 (Fpr2 reverse for WT – CGGAATCCAGCTACCCAAATC) and GB4 (Fpr2 reverse for KO –ATAACCTTCGGGCATGGCACT). The F1/B11 pair produced a band of 233 bp from the wild type allele, whereas F1 and GB4 produced a band of 351 bp for the allele of Fpr2/3 KO mice. A double band identified the heterozygous mice.

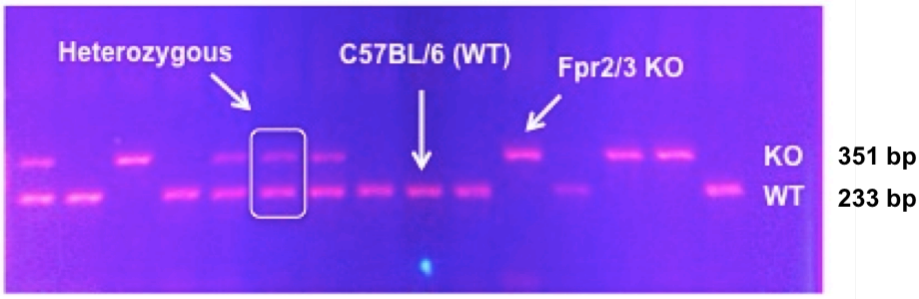


Figure 12: Genotyping of Fpr2/3 KO and C57BL/6 WT mice.
Ear clips DNA were removed from mice and screened by PCR. The genotyping was confirmed by southern blotting analysis. The F1 (Fpr2 forward), B11 (Fpr2 reverse for WT) and GB4 (Fpr2 reverse for KO) primers were used to identify the wild type allele (F1/B11 pair at 233 bp), the Fpr2/3 KO allele (F1/GB4 pair at 351 bp) and heterozygous mice (double band). Example of blot of 15 different mice is shown.

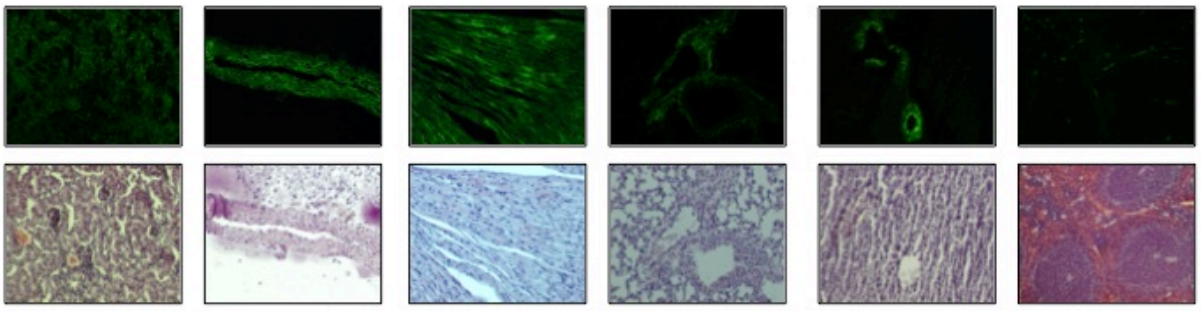
3.2 GFP expression of tissues harvested from Fpr2/3 KO mice.

The natural fluorescence of tissues harvested from the Fpr2/3 KO mice was identified and quantified by immunohistochemistry.

The tissues that exhibited GFP positivity were: aorta, heart, lung, spleen, liver and kidney (Figure 13 A). Using the imaging software ImageJ, the extent of GFP expression was also quantified finding the kidney as the tissue with the highest expression of the green fluorescent protein. Thus, taking the kidney as 100% of GFP expression, green fluorescence protein of the other tissues was quantified and compared as shown in Figure 13 bottom panel.

It is noteworthy to mention that to quantify GFP expression across all tissues identical conditions were kept: they were all stained with hematoxylin and eosin and observed using a 20x magnification applying an exposure of 56 milliseconds to the blue light.

A.



B.

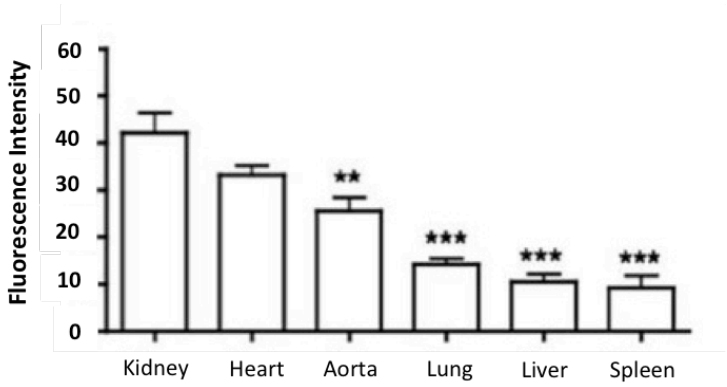


Figure 13: GFP expression in tissues harvested from Fpr2/3 KO mice.
(A) Natural fluorescence of tissues (from left: kidney, aorta, heart, lung, liver and spleen) harvested from the Fpr2 KO mice. The tissues were stained with haematoxylin and eosin and observed using a 20x magnification and an exposure of 56 seconds to the blue light. (B) Using the imaging software ImageJ the GFP expression was quantified in terms of fluorescence intensity. Data were calculated taking the values of the kidney as 100%. Data are expressed as mean ± SEM of at least 5 different slice of each tissue harvested from 6 to 15 mice (**p<0.01. ***p<0.001 *vs.* kidney).

3.2.1 Auto-fluorescesce of WT tissues: validation of GFP expression in the Fpr2/3 KO mouse

To validate the specificity of the green fluorescence of the Fpr2/3 KO mice the fluorescence of WT mice was also observed subjecting tissue slices to the same condition used for the Fpr2/3 KO mice (20x, 56milliseconds). No fluorescence was detected in any of the WT tissue samples (Figure 14).

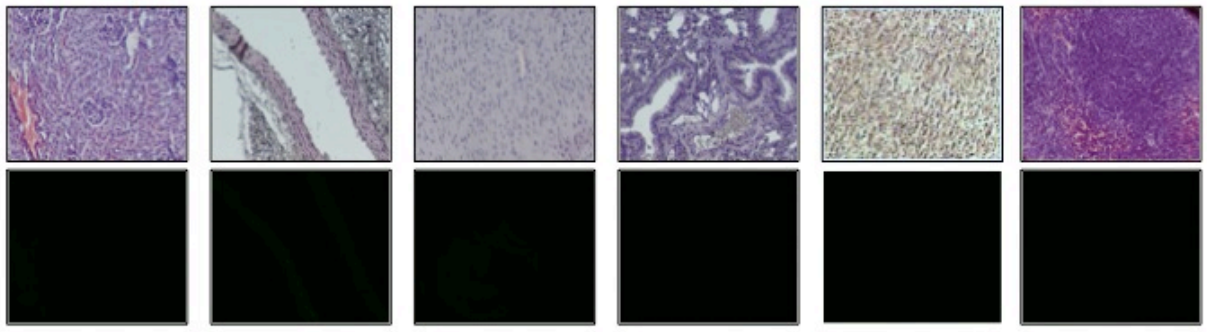


Figure 14: Green fluorescence of tissues harvested from WT mice.
Kidney, Aorta, Heart, Lung, Liver and Spleen (From Left to Right) harvested from WT mice were analysed using a 20x magnification after 56sec of exposure to the blue light. Pictures are representative of at least 5 different slice of each tissue harvested from 6 to 15 mice.

3.2.2 HRP staining with the anti mouse Fpr2 antibody

To compare – at least qualitatively – expression of Fpr2 in WT mice with the results obtained, and presented above for the GFP expression in the Frp2/3 KO tissues, an antibody against mouse Fpr2 (Santa-Cruz) was also used in WT mice. The antibody was tested in two tissues, selected because of their different degree of fluorescence, the kidney (Figure 15) and the lung (Figure 16).

The HRP staining was performed using different concentrations of the primary antibody. Examples of antibody titrations are shown. Negative controls for the staining procedures were also conducted, omitting the primary antibody or staining in the absence of any antibody (Panels A and B in both Figure 15 and Figure 16). Another important control for the specificity of the primary antibody was the use Fpr2/3 KO mice, which tissues I tested only with the optimal dilution (1:200 for Kidney and 1:100 for Lung). The anti-Fpr2 antibody did not give any staining in the null mouse tissue samples (Panels F,G and H of Figures 15 and 16).

That the anti-Fpr2 antibody worked in the WT mice and not in the Fpr2/3 KO emphasized the specificity of the antibody for the Fpr2 receptor. Therefore, the results obtained with the GFP staining in tissues prepared from Fpr2/3 KO mice could be considered genuine, and the protocol used further, since there was at least a qualitative match and validation with WT tissues using the anti-Fpr2 antibody.

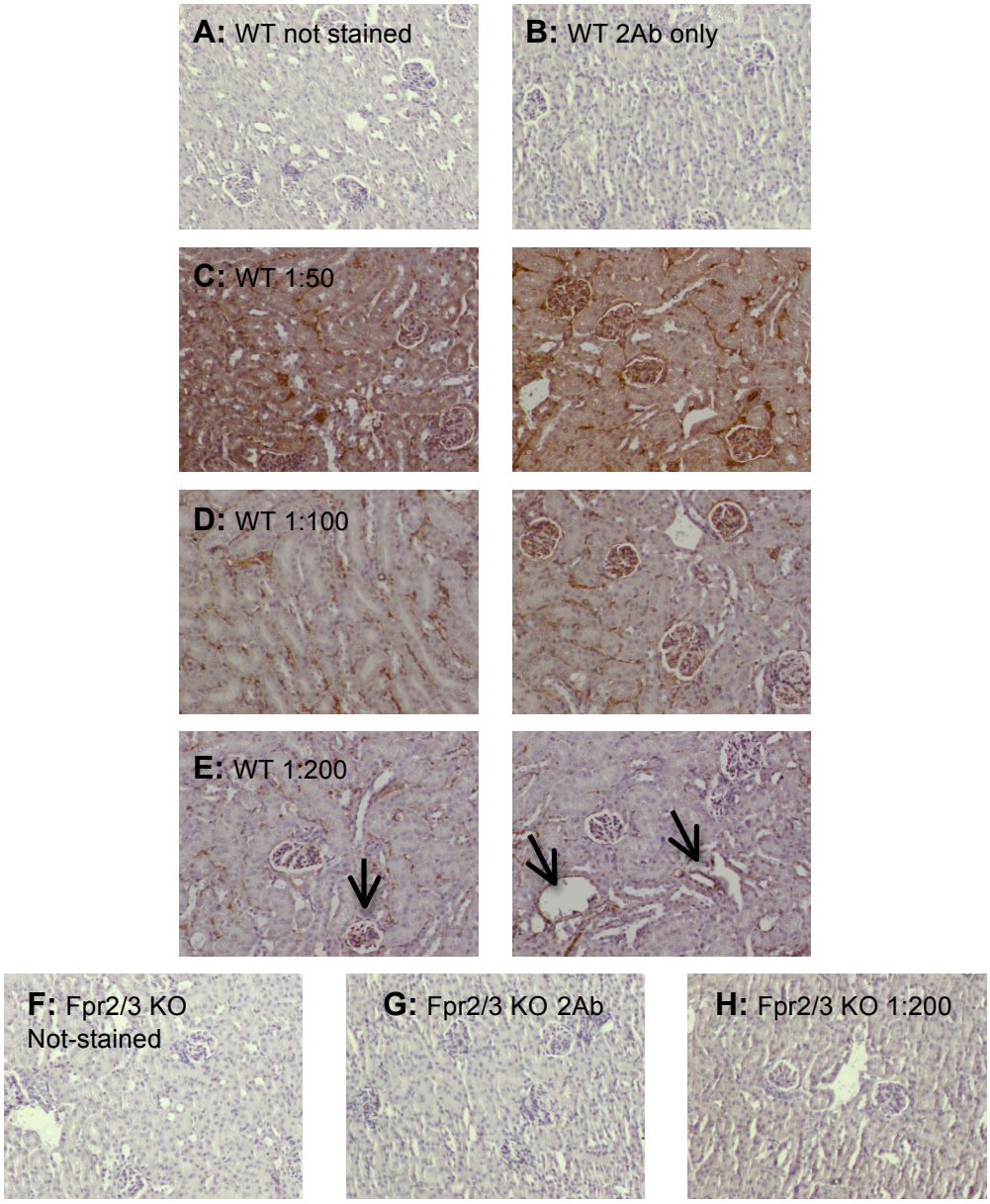


Figure 15: Fpr2 staining on kidneys harvested from WT and Fpr2/3 KO mice.
The staining with the anti-mouse Fpr2 antibody was performed on WT mice at different dilutions of 1:50 (C), 1:100 (D) and 1:200 (E). Images of WT tissue not-stained (negative control; A) and stained with the 2°Ab (B) are shown. As a further control the staining was performed in Fpr2/3 KO tissue samples. Picture of not-stained (F), and stained with the 2° antibody (G) and the Fpr2 antibody at 1:200 dilutions (H) are shown. Images are representative of at least 6 different slices of each tissue harvested from 5 to 15 mice.

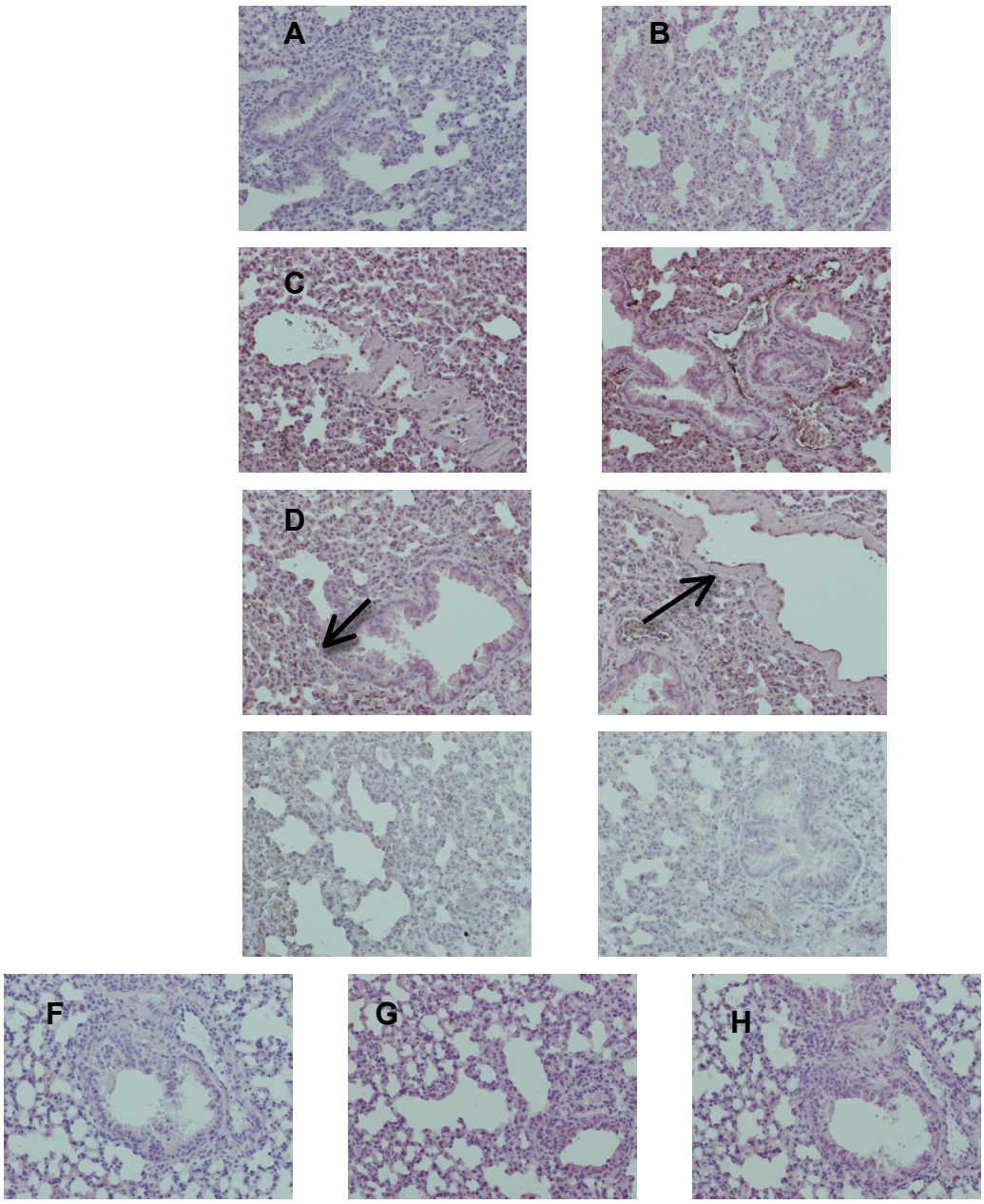


Figure 16: Fpr2 staining on lungs harvested from WT and Fpr2/3 KO mice.
The staining with the anti-mouse Fpr2 antibody was performed on WT mice at different dilutions of 1:50 (C), 1:100 (D) and 1:200 (E). Images of WT tissue not-stained (negative control; A) and stained with the 2°Ab (B) are shown. As a further control the staining was performed in Fpr2/3 KO tissue samples. Picture of not-stained (F), and stained with the 2° antibody (G) and the Fpr2 antibody at 1:200 dilutions (H) are shown. Pictures are representative of at least 6 different slices of each tissue harvested from 5 to 15 mice.

3.2.3 Western Blotting Analyses.

We have also established whether Western blotting could also be a valid method to 'chase' GFP expression, hence potential modulation of Fpr2/3 gene activity. To do this, we have tested different anti-GFP antibody:

1. Invitrogen – GFP ABfinity recombinant rabbit monoclonal (G10362)
2. Invitrogen – Anti-GFP chicken (A10262)
3. AbD Serotec – Goat Anti Green Fluorescent Protein (AHP975)
4. Clontecg – Living Colours A.v. Peptide Antibody (632377)

and tested them in bone marrow cells (Figure 17 A) and kidney's lysate harvested from the Fpr2/3 KO mice (Figure 17 A,B)

Kidney was used as the tissue with the highest expression of the GFP.

As a positive control and, to test the validity of the antibodies, we have used HEK-293 cells stable transfected with the green fluorescence protein (Figure 17 A). In other experiment we also used kidney of KO mice treated with LPS, an inflammatory stimuli able to induce Fpr2/3 (Figure 17 B). Unfortunately we have been not able to detect the presence of GFP in any of the Fpr2/3 KO cells and tissues using this technique.

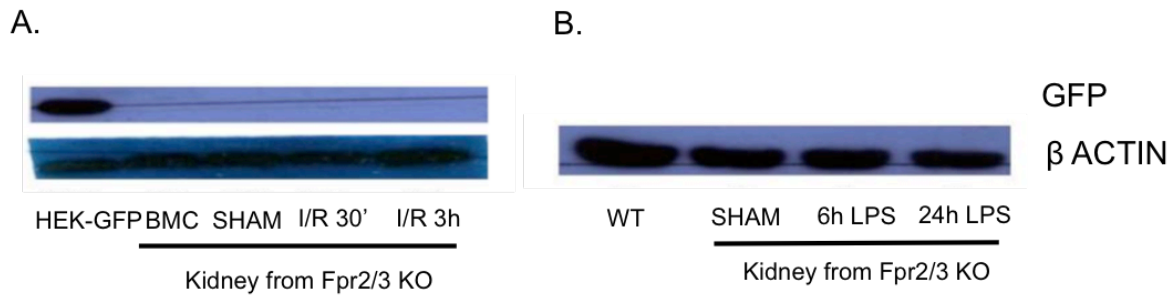


Figure 17: Western Blot analysis.

Representative blots of HEK-GFP cells, Fpr2/3 KO Bone Marrow Cells (BMC) and Fpr2/3 KO Kidney lysates from sham surgery mice or mice after 30 min or 3h or ischemia/reperfusion injury, incubated with anti-GFP Ab (1:1000) and beta-actin antibody (A). Beta-actin of kidney lysate from sham surgery WT and Fpr2/3 KO mice or Fpr2/3 KO treated for 6h and 24h with LPS (10mg/kg i.p). No GFP signals were detected (B).

3.2.3 GFP expression on cells of Fpr2/3 KO mice

FPR2/ALX is expressed on a variety of immune cells, including neutrophils, mononuclear cells and lymphocytes. To validate the GFP expression in these cell types blood, bone marrow derived cells and peritoneal cells of Fpr2/3 KO mice were analysed.

Blood was collected by cardiac puncture from both WT and Fpr2/3 KO mice and, after lysis of red blood cells, was analyzed by flow cytometry. To monitor the natural GFP fluorescence the FL-1 channel was used and to amplify the GFP signal, an intracellular staining with an anti-GFP antibody was performed. As shown in Figure 18, neutrophils exhibit ~6% of natural green fluorescence that is amplified up to ~20% value using an anti-GFP antibody at a final dilution of 1:50 (titration of the antibody was performed as showed in figure 18 B). Performing the Ly6G staining procedure the GFP fluorescence was covered and lost. To avoid this problem neutrophils were gated in the FSC/SSC plot (Figure 18 A) and GFP expression of this specific population was then quantified.

GFP expression in bone marrow cells was detected without the use of specific anti-GFP antibodies. Indeed, Fpr2/3 KO mice showed ~14% of GFP positive cells in the FL-1 channel (against a background of ~2% in WT mice - Figure 19 A). Deeper phenotyping of the FL-1 positive population with markers for macrophages and neutrophils revealed that ~25% of events corresponded to F4/80+ve cells whilst ~40% Ly6G+ve cells could be found (Figure 19 B).

Bone marrow derived cells were also differentiated into macrophages over a 5-day culture protocol in L929 conditioned medium. Fpr2/3/GFP expression was monitored over this time course (Figure 20 B,C). Already at Day 1, cells harvested from Fpr2/3 KO mice (Figure 20 A-blue line) showed a detectable GFP+ve population, which was clearly not present in WT mice (Figure 20 A-red line). As can be seen in Figure 20 B, the percentage of GFP positive cells remained unchanged up to Day 3 whilst it underwent a major increase on Day 4 (Figure 20 D,E,F). A more gradual increase in GFP expression could be monitored in terms of MFI units, i.e. intensity of GFP expression at the single cell level. Indeed, the median of MFI united changed from a value of 5 at Day 2 to a value of ~20 at Day 5 (Figure 20 C).

Finally, peritoneal cells were also studied. Out of the total peritoneal cells harvested from the Fpr2/3 KO mice, ~5% of GFP positive cells (with a 10 units for MFI) and ≥50% GFP positive cells (with a median intensity of 25 units) were revealed using an anti-GFP antibody (Figure 21 A,B). Further detailing with specific cell markers indicated presence of ≤1% of Ly6G positive cells in the pool of GFP positive peritoneal cells prepared from naïve cavities (Figure 21 C) whilst 30% of cells were F4/80 positive (Figure 21 D). WT mice were used as a negative control.

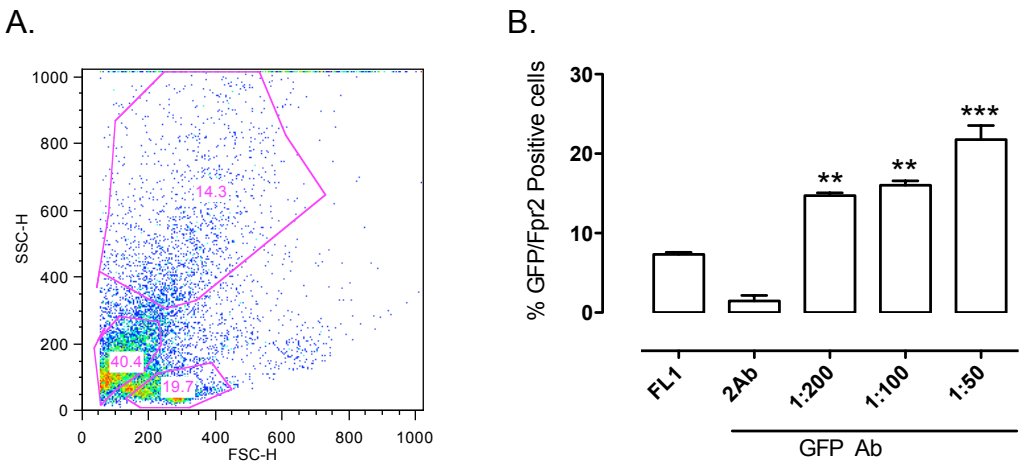
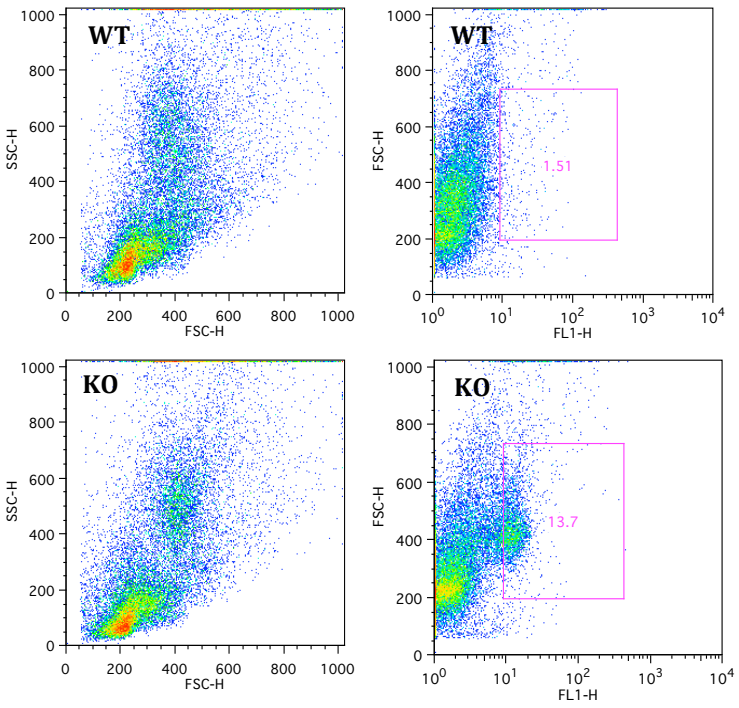


Figure 18: Fpr2/GFP expression in Neutrophils.

Whole blood was collected by cardiac puncture from Fpr2/3 KO mice and analysed by Flow Cytometry after lysis of the red blood cells. (A) Representative FSC/SSC plot. (B) Analysis of the gated neutrophils, revealed a 6% (MFI 6%) of natural green fluorescence that was amplified to a 20% value using an anti-GFP antibody at 1:50 concentration. Data, analysed with FlowJo software and reported as % of positive cells, are expressed as mean ± SEM of 10 to 20 mice. * $p<0.05$, **$p<0.01$, *** $p<0.001$ vs FL1.

A.



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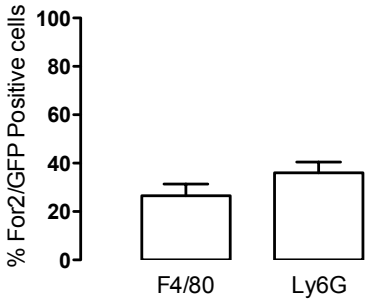

Figure 19: Fpr2/GFP expression in Bone Marrow Cells.

Bone marrow cells collected from femurs and tibia of WT and Fpr2/3 KO mice were analysed by flow cytometry. (A) Analysis of the FL-1 channel revealed an exclusive population of 13.7% GFP positive cells in Fpr2/3 KO mice. WT mice showed 1.5 % of FL-1 positive cells. (B) The Fpr2 KO/GFP population was characterised by 25% of F4/80 positive cells and 40% of Ly6G positive cells. Data have been analysed using the FlowJo software and are reported as % of positive cells. Data are expressed as mean ± SEM of 10 to 20 mice.

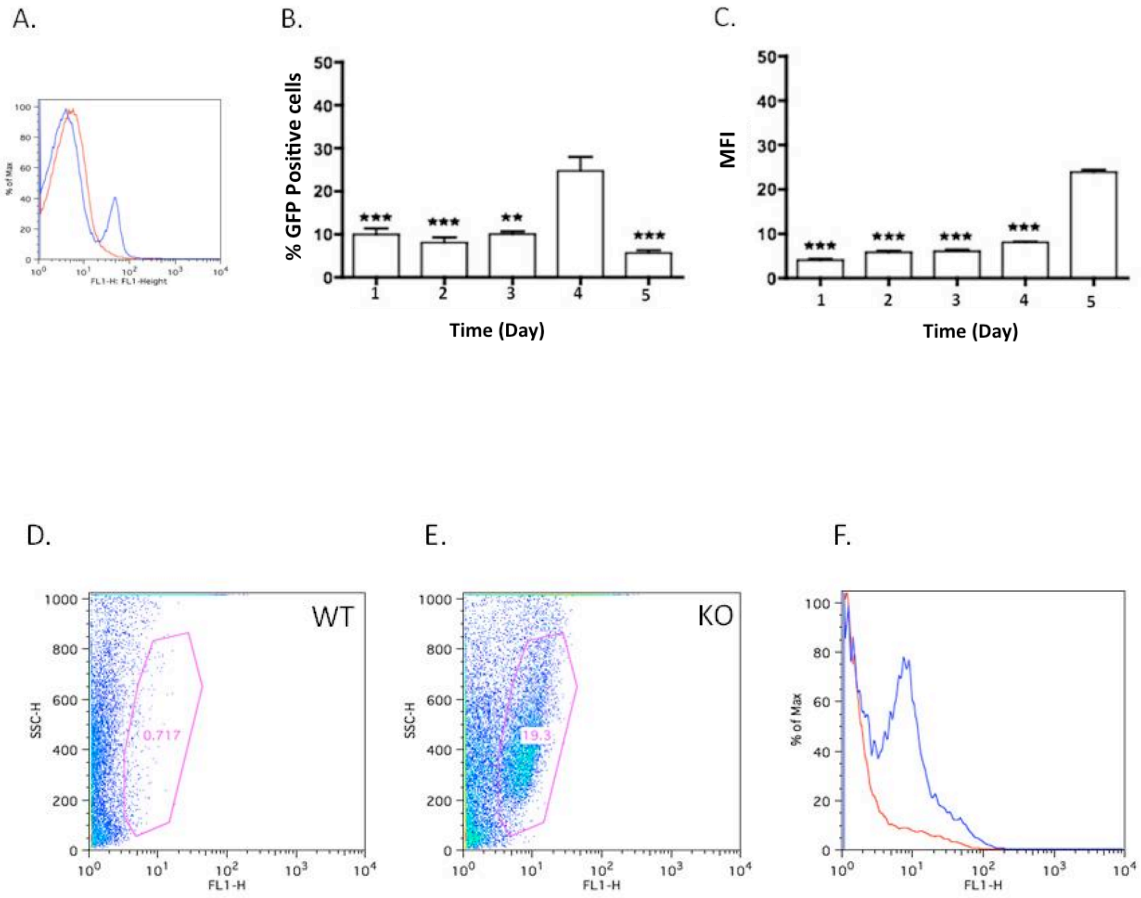

Figure 20: GFP expression in bone marrow derived macrophages.

WT and Fpr2/3 KO bone marrow cells have been collected and differentiated into macrophages for 5 days in L929 conditioned medium. (B,C) Monitoring of GFP expression, expressed in terms of % of positive cells (B) and MFI (C), during the 5 days of differentiation. (A) Fpr2/3 KO and not WT cells, were positive for the GFP already at day 1 and increase in numbers until day 4 (E,F). (D) GFP expression in WT mice also at day 4 is inexistent. Results, analysed with the FlowJo software, are reported as % of positive cells and as median fluorescence intensity (MFI) units for specific fluorescence. Data are expressed as mean ± SEM of 10 to 15 mice (**p<0.01, *** p<0.001 vs Day 4 in figure B and Day 5 in figure C).

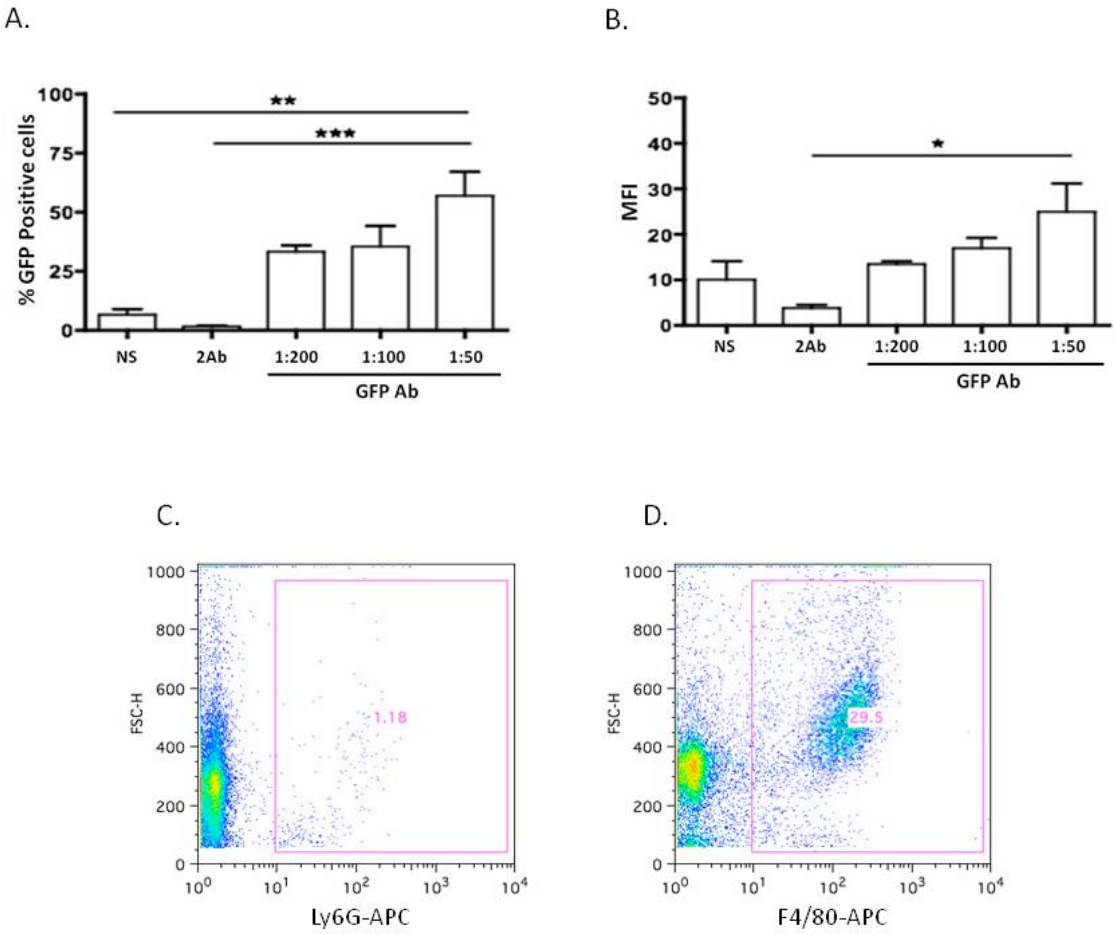

Figure 21: GFP expression in peritoneal cells.

Peritoneal cells from the cavity of Fpr2/3 KO were collected with a lavage of PBS and EDTA and analysed by flow cytometry. (A,B) Fpr2 KO peritoneal cells showed a 5% of FL-1 positive cells with a 10% of MFI increased up to 50% of GFP positive cells with a 25% of MFI using an anti-GFP antibody (1;50). Other concentrations of anti-GFP antibody were also used (1:200, 1:100). (C,D) The GFP positive population is composed by 1% of Ly6G positive cells and 30% of F4/80 positive cells. Results have been analysed using the FlowJo software and reported as % of positive cells and as median fluorescence intensity (MFI) units for specific fluorescence. Data are expressed as mean ± SEM of 10 to 15 mice (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3.2.4 Conclusion

FPR1 and FPR2/ALX cellular and tissue distribution is mirrored by their murine orthologues, suggesting the important information that they might maintain physiological roles and functions across the species (Fu, Karlsson et al. 2006). FPR1 and FPR2/ALX receptors are expressed on PMN and mononuclear cells, both myeloid and lymphocytes (Schiffmann, Corcoran et al. 1975, Showell, Freer et al. 1976, Aswanikumar, Corcoran et al. 1977, Zigmond 1977). Human and rodent FPR1 receptor display quiet a widespread distribution since it has been found, using immunohistochemical methods, in tissues that include epithelial cells (Rotrosen, Malech et al. 1987), endocrine cells (including follicular cells of the thyroid and cortical cells of the adrenal gland), platelets, liver hepatocytes and Kupffer cells, smooth muscle cells, brain, spinal cord and both sensory and motor neurons (Lacy, Jones et al. 1995, McCoy, Haviland et al. 1995, Le, Hu et al. 2000, Czapiga, Gao et al. 2005, Fu, Karlsson et al. 2006). The localization of FPR2/ALX has not yet been investigated, though it is expressed by endothelial cells (Fierro, Colgan et al. 2003) and epithelial cells (Leoni, Alam et al. 2013). However, the tissues distribution of the human FPR gene family members remains a partial unexplored field.

GFP expression was studied in Fpr2/3 KO tissues using fluorescence microscopy. Using this approach we were able to identify Fpr2/3 localization around the body: the quantification of the fluorescence revealed that the Fpr2/3 genes may be more active (or expressed by a higher number of positive cells) in the kidney, followed by the heart, aorta, heart, lung, liver and spleen (Figure 13,14). It was important to

corroborate the findings obtained by GFP fluorescence with the anti-Fpr2 staining in WT tissue (Figure 15,16).

GFP expression has also been studied in cells harvested from the Fpr2/3 KO mice. In the first 24 hours of the inflammatory reaction, the infiltration is predominantly composed by PMNs (Witko-Sarsat, Rieu et al. 2000, Kantari, Pederzoli-Ribeil et al. 2008). During on-going inflammatory reactions the profile of infiltrated cells change from PMNs to monocyte and macrophages. The number of the latter cells increased more slowly becoming the major cells type between 48 and 72 hours (Bellingan, Caldwell et al. 1996, Kantari, Pederzoli-Ribeil et al. 2008). The great relevance of neutrophils and macrophages in the resolving phase of an acute inflammatory process (Nathan 2002, Luster, Alon et al. 2005) led us to focus our work on these two populations. To monitor the GFP expression of neutrophils and macrophages we used the flow cytometry; the FL-1 channel was used for the green natural fluorescence and to amplify the signal, an intracellular staining with an anti-GFP antibody was performed.

We started off by trying to quantify the GFP expression in the whole blood however, even when we added an anti-GFP antibody, we could not obtain a strong enough fluorescent signal. Then, we stained neutrophils with the Ly6G marker in a mixed population of blood cells after removal of erythrocytes by lysis; however, the double staining masked the GFP fluorescence. To avoid this problem we used the scatter characteristics to gate the neutrophils in the FSC/SSC plot and quantify the GFP expression of this population (Figure 18 A). Using this method, the neutrophils of the Fpr2/3 KO mice exhibited ~5% of GFP +ve signal (with a value of MFI units of

6), and this was increased to 20% (with MFI units of 9%) value using an anti-GFP antibody at 1:50 concentration (Figure 18 B).

I have also tried other techniques and protocols for separation and analysis of neutrophils from the whole blood. We tried different protocols with Histopaque of different density, the protocol that we used for the isolation of human neutrophils or we attempted to sort neutrophils from the whole blood using a sorting machine. None of these protocols gave positive results. I conclude that the more appropriate method is using a mixed population of cells (e.g. peripheral blood cells after lysis of the red blood cells), gating PMN using their forward and side scatter profile, and analyse their degree of GFP expression. All in all I am not fully confident that this protocol can be consistently used for this cell type.

We obtained better results when macrophage expression was investigated. Firstly, we started with bone marrow cells, which gave a clear signal in the FL1 channel when prepared from Fpr2/3 KO mice (~10% of GFP positive cells) (Figure 19). Deeper phenotyping of the FL-1 positive population with markers for macrophages and neutrophils revealed that ~25% of events corresponded to F4/80+ve cells whilst ~40% Ly6G+ve cells could be found (Figure 19 B). Following analysis on the presence of GFP staining in the bone marrow, we moved on to bone marrow derived macrophages, prepared over a 5-day culture protocol (see Figure 10: bone marrow cells differentiation using L929 conditioned medium). Since it has been published that FPR2/ALX expression remains unchanged during monocyte differentiation into macrophages (Ye, Boulay et al. 2009) we monitored GFP expression daily. Already only after 1 day of differentiation the bone marrow cells

harvested from Fpr2/3 KO mice showed a GFP positive population which was obviously absent in WT mice. During the 5 days of differentiation, as can be seen in figure 20 B and C, the percentage of GFP positive cells remained unchanged during the first three day and had a significant increase on day 4. Instead, in terms of MFI, we detected a gradual increase. It changes from 5 at day 2 to 18 at day 5. On figure 20 D,E and F it is shown the FL-1 expression in WT (Figure 20 D, F-red line) and Fpr2/3 KO mice (Figure 20 E, F-red line) at day 4. It is evident that the FL-1 positive population is specific for the Fpr2/3 KO mice and that, during the differentiation into macrophages the GFP positive cells increase in terms of number (Figure 20).

We have also analysed peritoneal macrophages. The peritoneal cavity is a unique compartment within which a variety of immune cells reside, and from which macrophages are commonly collect for functional studies. Peritoneal cells harvested from the Fpr2/3 KO mice showed 5% of FL-1 positive cells with a 10% of MFI and 50% of GFP positive cells with a 25% of MFI using an anti-GFP antibody (Figure 21 A,B). In the peritoneal cells we founded 1% of Ly6G positive cells (Figure 21 C) and 30% of F4/80 positive cells (Figure 21 D).

We also tried do quantify the GFP expression by Western blotting using different anti-GFP antibody. Unfortunately we have been not able to detect the presence of GFP in any of the Fpr2/3 KO cells and tissues using this technique (Figure 17).

In conclusion we have monitored the pattern of expression of Fpr2/GFP in murine tissues and cells. We are not confident that we will be able to quantify the GFP

expression as a report for Fpr2/3 gene promoter activity but we will use these techniques to localize Fpr2/3 in our model. Also, Fpr2/3 is expressed in the organs of our interest (heart, lung and kidney) and in infiltrating cells that characterize the inflammatory response during acute myocardial injury, all in all increasing our interest in studying the role of this receptor in AMI in the heart and in secondary injury organs.

CHAPTER 4: RESULTS

4.1 Echocardiography in WT and Fpr2/3 KO sham mice: evaluation of left ventricle function

To confirm that WT and Fpr2/3 KO mice have the same left ventricle function in physiological condition echocardiography was performed in sham mice. Both WT and Fpr2/3 KO mice showed the same heart function and contractility as expressed in terms of heart rate, interventricular septal thicknesses, left ventricle internal dimensions, posterior wall thicknesses, ejection fraction, fractional shortening and posterior wall thickening (Table 1).

	HR (bpm)	EF (%)	FS (%)	IVS;d (mm)	LVID;d (mm)	LVPW;d (mm)
WT	563.60±35.61	76.66±3.56	44.76±3.35	0.69±0.03	3.72±0.21	0.63±0.07
Fpr2 KO	552.40±81.77	75.24±3.21	43.40±2.93	0.75±0.08	3.69±0.18	0.70±0.12
	IVS;s (mm)	**LVID;s (mm)**	**LVPW;s (mm)**	**LV Vol;d**	**LV Vol;s**	**MV E/A**
WT	1.33±0.11	2.06±0.22	1.29±0.09	59.15±7.90	14.01±3.46	1.59±0.29
Fpr2 KO	1.38±0.10	2.09±0.16	1.29±0.17	58.05±6.8	14.45±2.71	1.52±0.28

Table 1: Left ventricle (LV) function of sham WT and Fpr2/3 KO mice.
Echocardiography data of WT and Fpr2 KO mice in normal condition with correlated heart rate (HR) value. At diastole (d) and systole (s): LV interventricular septal thicknesses (IVS), LV internal dimensions (LVID), Posterior wall thicknesses (LVPW), Ejection fraction (EF), Fractional shortening (FS), Posterior wass thickening (PWT). Data (mean ± SEM) of 6 mice. *P<0.05 between WT and Fpr2 KO.

4.2 Role of Annexin A1 and Formyl Peptide Receptors in Acute Myocardial Infarct

AnxA1 (1 µg per mouse ~ 27 pmol (Gavins, Dalli et al. 2007)) was injected intravenously in WT mice and the infarct size was quantified. The AMI procedure led to necrosis of ~55% of the area at risk (AAR) (Figure 22 A). Administration of AnxA1 before LADCA occlusion afforded significant cardioprotection (~30% reduction of infarct size, $p<0.01$) as compared to vehicle (PBS). AnxA1 administration at the beginning of reperfusion afforded a much lower degree of protection (~10%) (Figure 22 B,C). To evaluate the involvement of the Formyl Peptide Receptor (Fpr) 1 and 2 in AnxA1 mediated cardioprotection, similar experiments were performed in Fpr1 and Fpr2/3 KO mice using an i.v injection of AnxA1 prior to ischemia. Deletion of Fpr1 did not significantly alter the cardioprotective effect of AnxA1, whilst the protein less effective in Fpr2/3 KO mice ($p<0.05$) (Figure 23).

Analysis of mRNA expression in hearts of WT mice after AMI revealed a marked increase in Fpr2 mRNA during ischemia (Figure 24 A) and an increase in AnxA1 mRNA during reperfusion (Figure 24 B). Upregulation of Fpr1 mRNA occurred in both ischemia and reperfusion (Figure 24 C). Exogenous AnxA1 administration at the beginning of ischemia also induced an upregulation in the expression of Fpr2 mRNA after AMI ($p<0.01$ *vs*. Vehicle) (Figure 24 D).

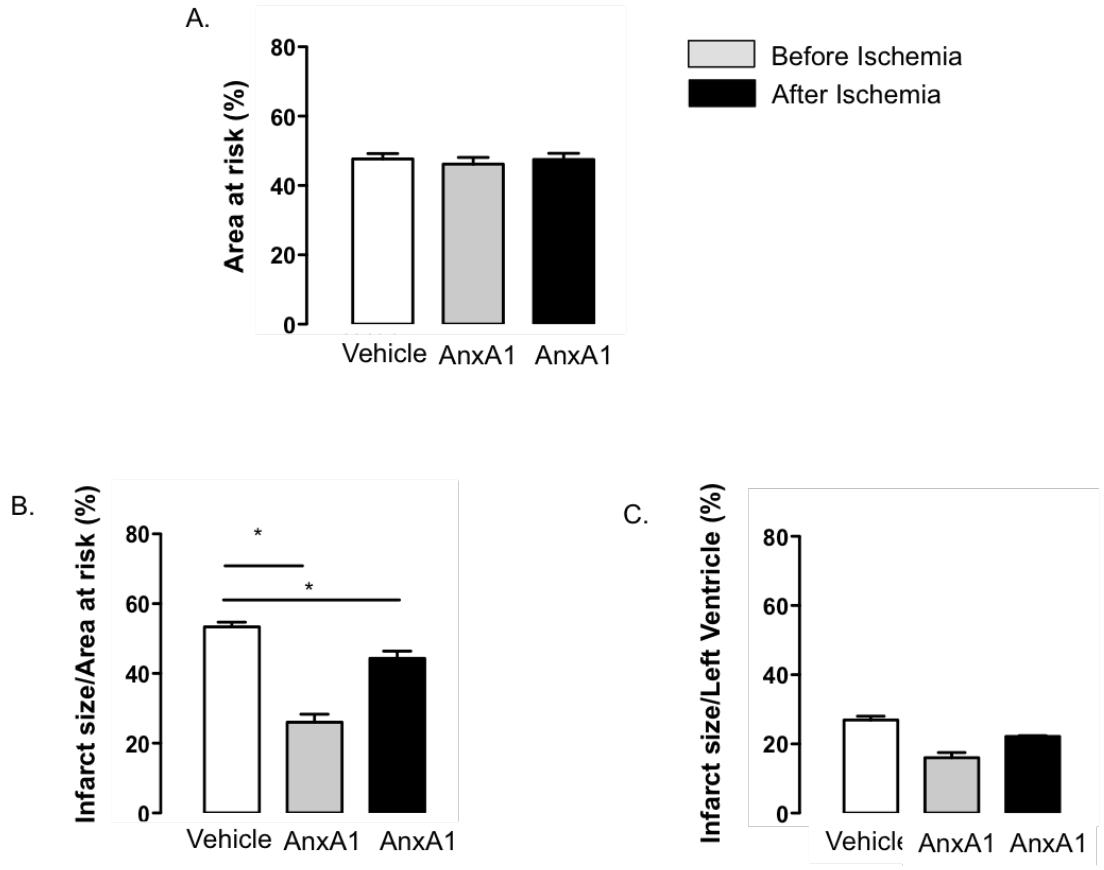

Figure 22: AnxA1 cardioprotection in vivo.

WT mice were injected intravenously with vehicle (PBS) or AnxA1 as shown. AMI procedure of 30+90 min I/R was applied, through occlusion of the LADCA, and area at risk and infarct size analysed. (A) Occlusion of the artery blocked the oxygen to a ~55% of the left ventricle (Area at risk - AAR). Necrosis of this area are is referred as infarct size (B) and the left ventricle (C). I.v. injection of AnxA1 (1 µg) before ischemia afforded significant cardioprotection (~30% reduction of infarct size, $p<0.01$) as compared to vehicle (PBS). AnxA1 administration at the beginning of reperfusion afforded a less degree of protection (~10%). Data are expressed as mean ± SEM of 8 to 10 mice per group.

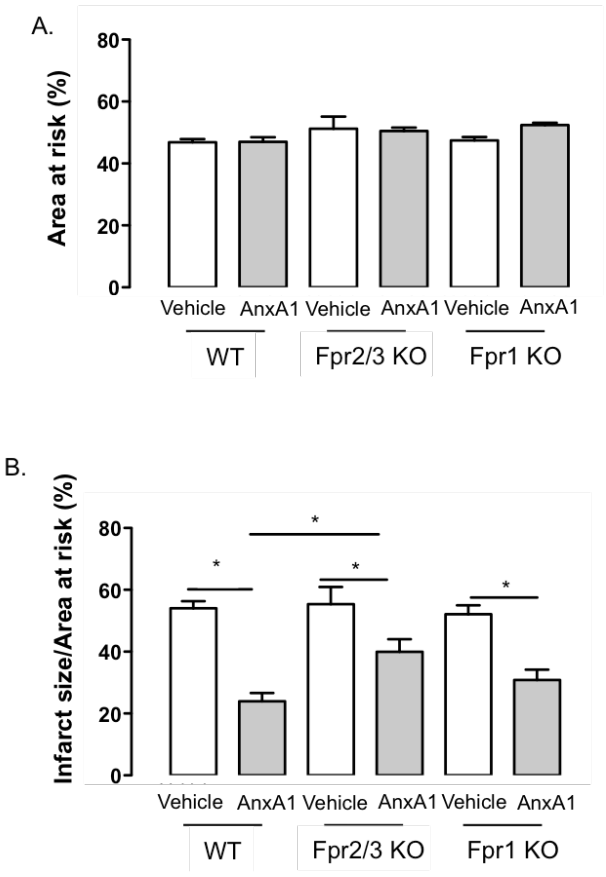

Figure 23: Role of Fprs on AnxA1 cardioprotection in vivo.

WT, Fpr1 and Fpr2/3 KO mice were subjected to 30 min of ischemia through occlusion of the LADCA and 90 min of reperfusion. All mice were injected intravenously with vehicle (PBS) or AnxA1 (1 µg) prior ischemia. At the end of reperfusion the area at risk and the infarct size were analysed. (A) LADCA occlusion induced ~55% necrosis of the AAR in WT, Fpr1 and Fpr2/3 KO mice. (B) AnxA1 cardioprotective activity decreased in both Fpr2/3KO and Fpr1 KO mice ($p<0.05$). (C) The decrease of infarct size induced by AnxA1 was significantly less in the Fpr2/3 KO mice compared to WT mice ($p<0.05$). Data are showed as mean ± SEM of 8 to 10 mice per group.

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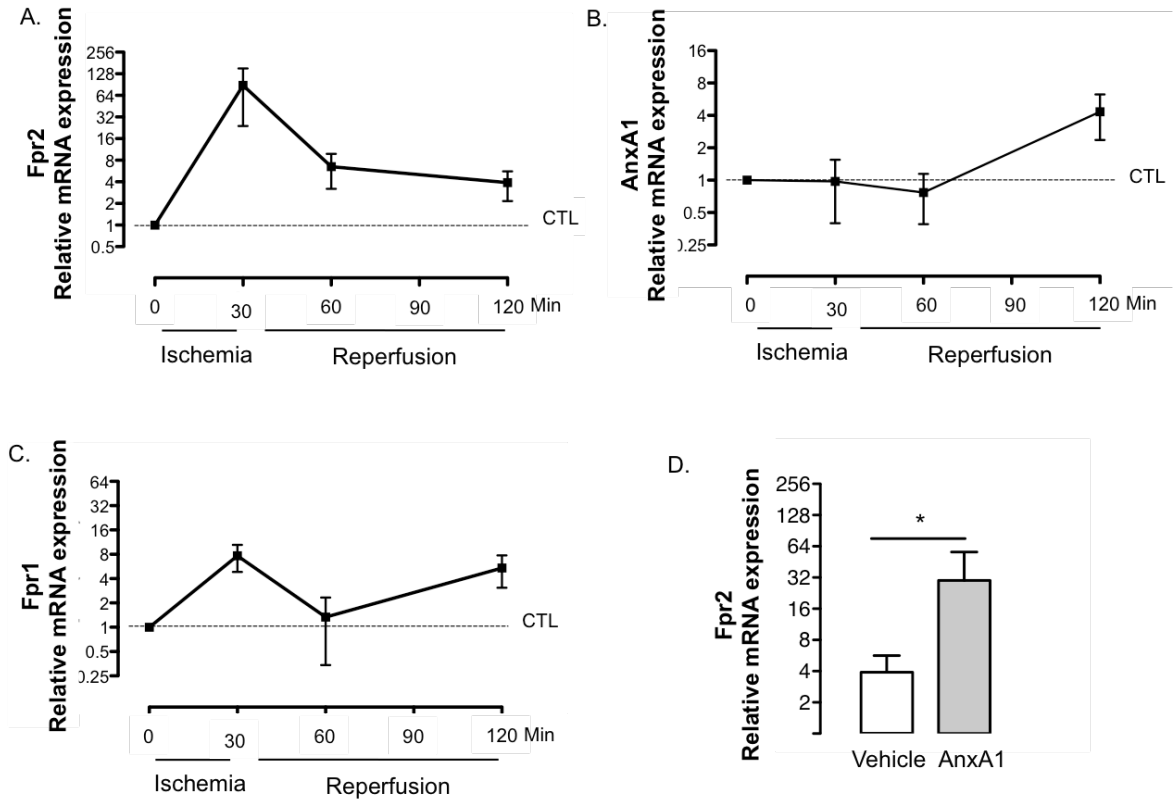


Figure 24: AnxA1, Fpr2 and Fpr1 mRNA modulation during AMI.

WT mice were subjected to AMI. At indicated time points hearts were collected and RNA and cDNA extracted and analysed with specific primers. mRNA regulation of (A) Fpr2, (B) AnxA1 and (C) Fpr1 is shown. (D) WT mice were treated i.v. with vehicle (PBS) or AnxA1 (1 µg) prior ischemia. After 30+90 min I/R Fpr2 mRNA upregulation was higher in mice treated with the protein ($p<0.01$). According to the relative quantification method the response produced in control mice (no AMI) was taken as 1 and data represent the upregulation (>1) or downregulation (<1) of mRNA expression. GAPDH was used as endogenous control. Data are showed as mean ± SEM of 8 to 10 mice per time point.

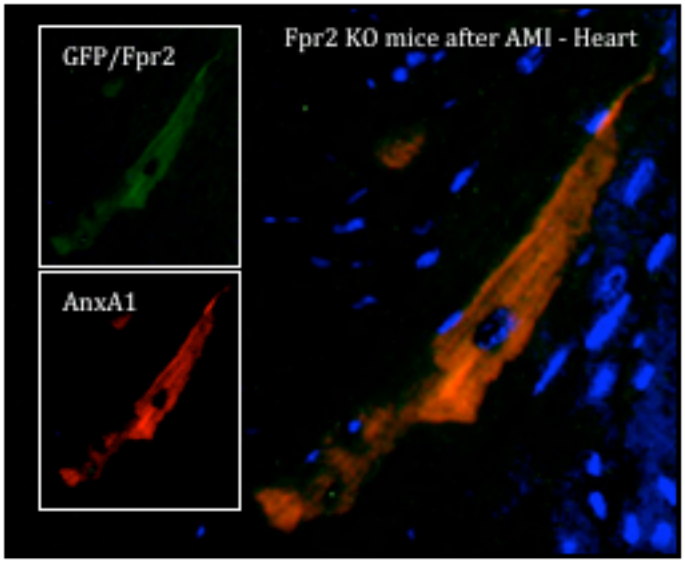
4.2.1 The role of Fpr2/3 in AnxA1 cardioprotection

Immunofluorescence of Fpr2/3 (GFP) and AnxA1 showed that the Fpr2/3 and Annexin A1 are expressed, and co-localized, in cardiomyocytes and in the neutrophils (Ly6G positive cells) of heart subjected to acute myocardial infarct (Figure 25).

To evaluate the damage induced by AMI and the role of AnxA1 during this patology, plasma troponin I levels and caspase 3 activity in the heart were evaluated. Troponin I is a contractile regulatory protein that controls calcium-mediated interactions between actin and myosin in cardiac and skeletal muscles, and, importantly, is released into circulation in response to myocardial necrosis whereupon it can be used as a clinical biomarker of cardiac injury. Caspase 3 is a member of the cysteine-aspartic acid protease family. Caspases exist as inactive proenzymes that undergo proteolytic processing to produce two subunits that dimerize to form the active enzyme. Caspase 3 activation plays a central role in cell apoptosis.

AMI was able to induce Troponin I ($p<0.01$) in WT mice. The levels were significantly decreased ($p<0.01$) by an i.v injection of AnxA1 (1 µg) prior too ischemia (Figure 26 A). The effect of AnxA1 was lost in Fpr2/3 KO mice ($p<0.01$ WT AnxA1 AMI *vs.* KO AnxA1 AMI) (Figure 26 A). Also Caspase 3 activity was induced by AMI ($p<0.001$) and decreased after AnxA1 injection (Figure 26 B) only in WT mice ($p<0.001$) and not in Fpr2/3 KO mice ($p<0.001$ WT AnxA1 AMI *vs.* KO AnxA1 AMI) (Figure 26 B).

To study the inflammatory response the levels of the neutrophils chemokine KC and TNFα were evaluated. AMI significantly increased the levels of KC ($p<0.001$) that were decreased by AnxA1 ($p<0.001$) in WT and KO mice (Figure 27 A). AnxA1 injection also decreased the levels of TNFα, in this case only in WT mice ($p<0.05$) and not in KO mice ($p<0.001$) (Figure 27 B). Interesting the Fpr2/3 KO mice had a decrease ($p<0.05$) of the cardioprotective AnxA1 after AMI (Figure 28).



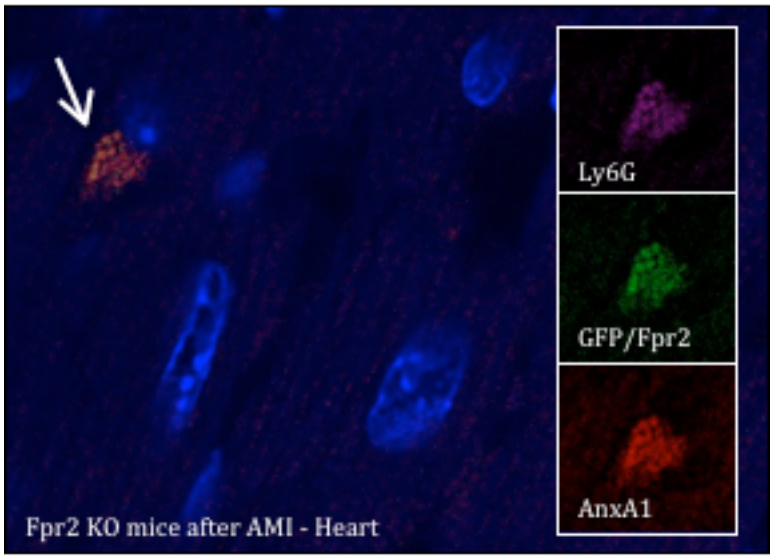


Figure 25: Immunofluoresce staining of heart of Fpr2/3 KO mice after AMI.
Hearts harvested from Fpr2/3 KO mice after AMI and subjected to immunofluorescence after staining with the AnxA1 antibody (in red, 1:1000) and the Ly6G antibody (in purple, 1:1000). Slides were also exposed to the blue light to allow the detection of the Fpr2/3 receptors expressed by the natural GFP fluorescence. The tissues were observed using a 20x magnification after immunofluorescence compensation.

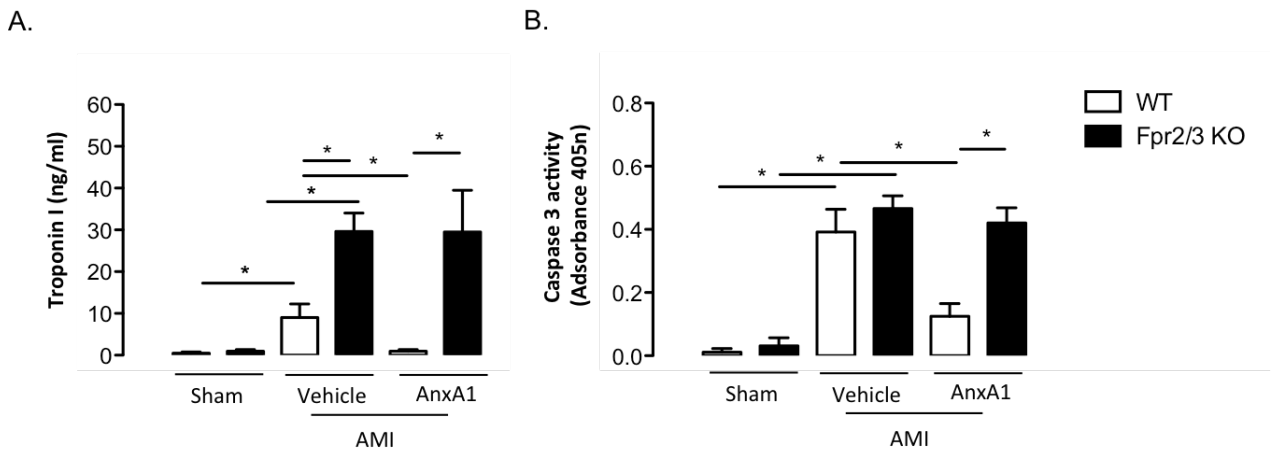


Figure 26: The cardioprotective effects of AnxA1 during AMI in WT and Fpr2/3 KO mice. AMI procedure of 30+90 min I/R, through occlusion of the LADCA, was applied on WT and Fpr2/3 KO mice. At the end of reperfusion plasma and heart were collected and analysed. In WT mice (white bar), AnxA1 (1 µg) administrated i.v prior ischemia, decreased the plasma levels of Troponin I ($p<0.01$) induced by AMI ($p<0.01$ *vs.* sham). The effect of AnxA1 was lost in Fpr2/3 KO mice (black bar) ($p<0.01$ AnxA1 WT *vs.* AnxA1 Fpr2/3 KO). (B) The role of AnxA1 on caspase 3 activity induced by acute myocardial infarct ($p<0.001$ *vs.* sham mice) in hearts was also evaluated. AnxA1 injection decreased the augmented levels of Caspase 3 in WT mice ($p<0.001$) and not in Fpr2/3 KO mice (black bar) ($p<0.001$ AnxA1 WT *vs.* AnxA1 Fpr2/3 KO). Data are expressed as mean ± SEM of 8 to 12 mice per group.

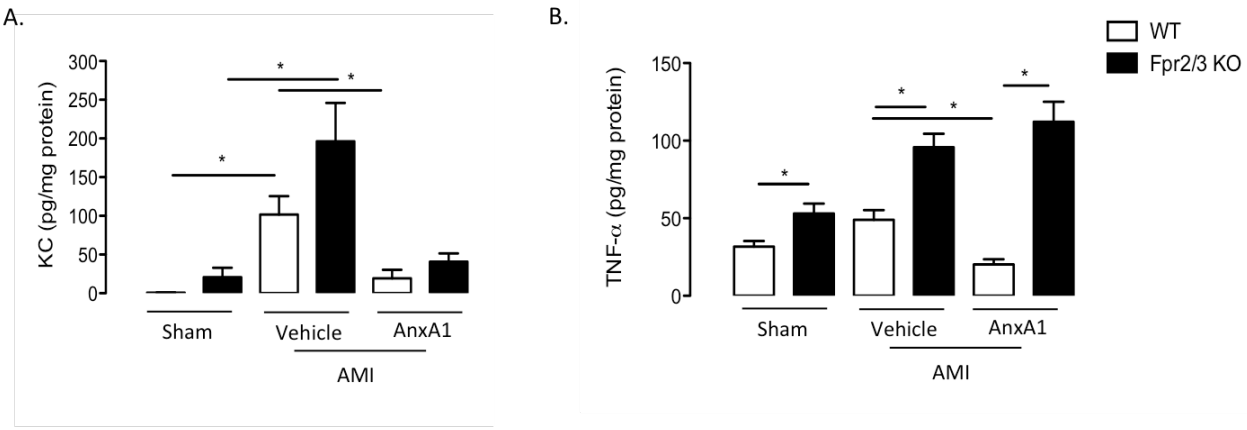


Figure 27: AnxA1 decrease the inflammatory response induced by AMI in the heart.
WT and Fpr2/3 KO were subjected to AMI after an i.v. injection of AnxA1 (1 µg) prior ischemia. Hearts were excised at the end of reperfusion and analysed. (A) AnxA1 decreased the levels of KC ($p<0.001$) induced by AMI ($p<0.001$) in WT (white bar) and Fpr2/3 KO mice (black bar). (B) AnxA1 injection also decreased the levels of TNFα ($p<0.05$) and the effect was lost in Fpr2/3 KO mice (black bar) ($p<0.001$). Data are mean ± SEM of 8 to 12 mice per group.

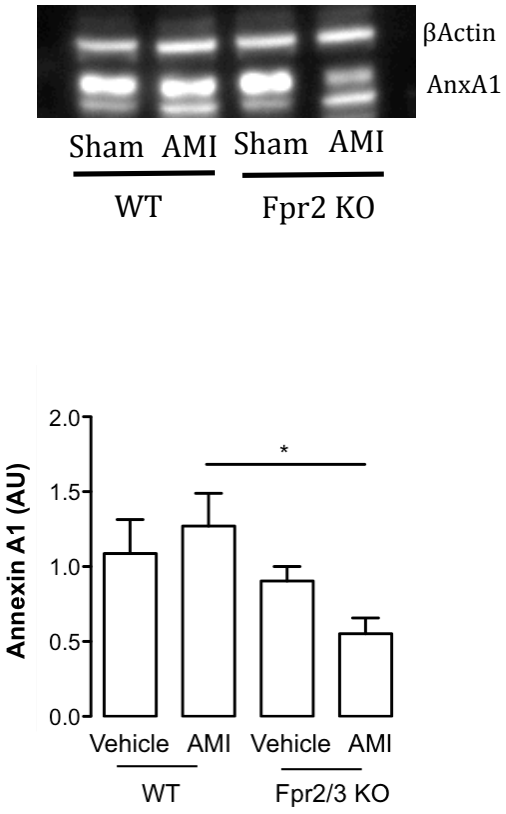


Figure 28: AnxA1 expression in WT and Fpr2/3 KO mice after AMI in the heart.
AnxA1 expression in hearts of WT and Fpr2/3 KO sham mice or subjected to acute myocardial infarct through 30 min of occlusion of the LADCA and 90 min of reperfusion was analysed. AnxA1 was decreased in Fpr2/3 KO mice after AMI ($p<0.05$ *vs.* WT AMI). Protein expression was monitored by western blot analysis using an AnxA1 antibody (1:1000, Cell Signalling) an anti-β-actin antibody (1:10,000; SigmaAldrich) as loading control. Representative blots from ≥3 experiments with distinct cell preparations are shown.

4.3 Effects of Acute Myocardial Infarct in Fpr2/3 KO mice

4.3.1 Survival and Infarct size of WT and Fpr2/3 KO mice

A significant proportion (~40%) of Fpr2/3 KO mice perished during the procedure of experimental AMI (Figure 29 A), and those that did survive up to 90 min post-reperfusion (Figure 29 A) exhibited a larger infarct size (~15%, $p<0.05$) than WT animals (Figure 29 C,D).

Congruently, Fpr2/3 KO mice showed an increase in plasma troponin I after AMI ($p<0.001$), and moreover, these levels were significantly higher in the Fpr2/3 KO mice than in WT animals ($p<0.05$) (Figure 30 A). Heart tissue from both WT and Fpr2/3 KO mice showed an increase in Caspase 3 activity after AMI ($p<0.001$) (Figure 30 B).

The expression of Fpr1, Fpr2 and AnxA1 mRNAs was up regulated in hearts of WT and Fpr2/3 KO mice after acute myocardial infarct (Figure 31 A). AnxA1 mRNA upregulation was significantly higher in Fpr2/3 KO mice compared with WT ($p<0.001$), whilst in contrast Fpr1 mRNA expression was upregulated similarly in the two different strains of mice (Figure 31 B,C). Interesting, the Fpr2 mRNA induction in the heart, during LADCA occlusion (Figure 24 A and Figure 32 A) were not connected with an increase in neutrophils infiltration, as seen by CXCL1 mRNA and KC quantification (Figure 32 C). During ischemia, the levels of KC were increased only in the plasma (Figure 32 B).

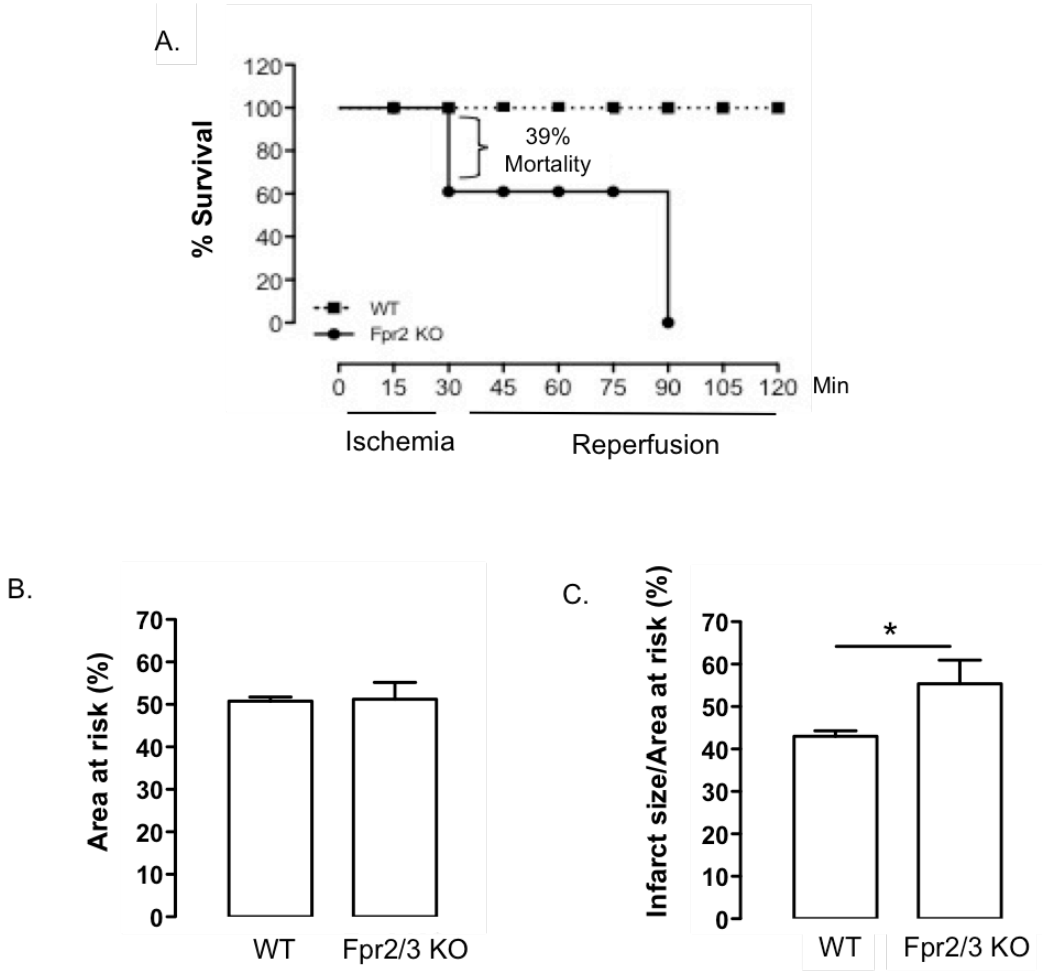


Figure 29: The impact of AMI on Fpr2/3 KO mice.

In WT and Fpr2/3 KO mice the LADCA was occluded for 30 min and re-opened for 90 min. Survival was monitored and recorded over all the AMI procedure. At the end of reperfusion the area at risk and infarct size were valuated. (A) 39% of Fpr2/3 KO mice and no WT mice died during the surgery. WT mice survived over all the reperfusion period (120h), Fpr2/3 KO were dying after 90 min. (B) AMI caused ~55% necrosis of the left ventricle (Area at risk) to both WT and Fpr2/3 KO mice. (C) Fpr2/3 KO mice showed a higher infarct size in comparison with WT mice ($p<0.05$). Infarct sizes are shown related to the area at risk. Data are expressed as mean ± SEM of 8 to 15 mice per group.

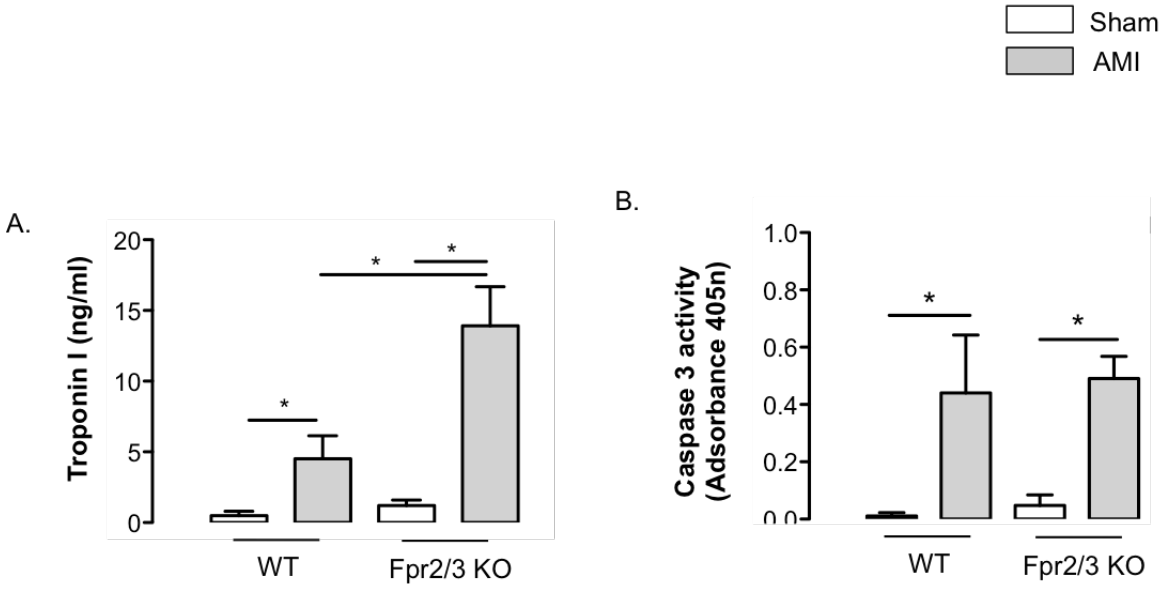


Figure 30: Troponin I expression and Caspase 3 activity in WT and Fpr2/3 KO mice.
AMI procedure characterised by 30+90 min I/R was applied to WT and Fpr2/3 KO mice. At the end of reperfusion plasma and hearts were collected and analysed. (A) WT ($p<0.05$) and Fpr2/3 KO mice ($p<0.001$) showed an increase in Troponin I expression in plasma after AMI. The increase in Troponin I levels (Sham mice *vs.* AMI mice) was significantly higher in the Fpr2/3 KO mice compared with WT mice ($p<0.01$). (B) Hearts from both WT and Fpr2 KO mice had an increase in Caspase 3 activity after AMI ($p<0.001$). Bars are representative of Sham mice (white bar) or mice where AMI has been induced (grey bar). Data are mean ± SEM of 8 to 15 mice per group.

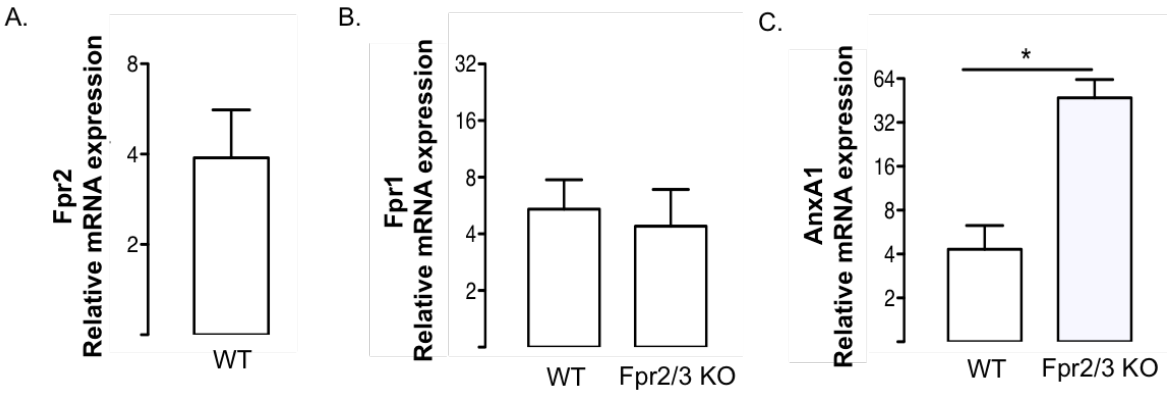


Figure 31: Upregulation of Fpr2, Fpr1 and AnxA1 mRNA expression in hearts of WT and Fpr2/3 KO mice after AMI.

mRNA analysis of WT and Fpr2/3 KO subjected to ischemia and reperfusion through sequential occlusion (30 min) and reopening (90 min) of the LADCA. Hearts were collected and RNA and cDNA extracted and analysed. (A, B) AMI induced an upregulation of Fpr2 in WT mice and Fpr1 in both WT and Fpr2/3 KO mice. (C) AnxA1 mRNA increased in both WT and Fpr2/3 KO mice with higher levels in Fpr2/3 KO mice ($p<0.001$). Using the relative quantification method the response produced in control mice (no AMI) was considerated as 1 and the data were expressed as an upregulation (>1) or downregulation (<1) of mRNA expression after AMI. GAPDH was used as endogenous control. Data are showed as mean ± SEM of 8 to 15 mice per group.

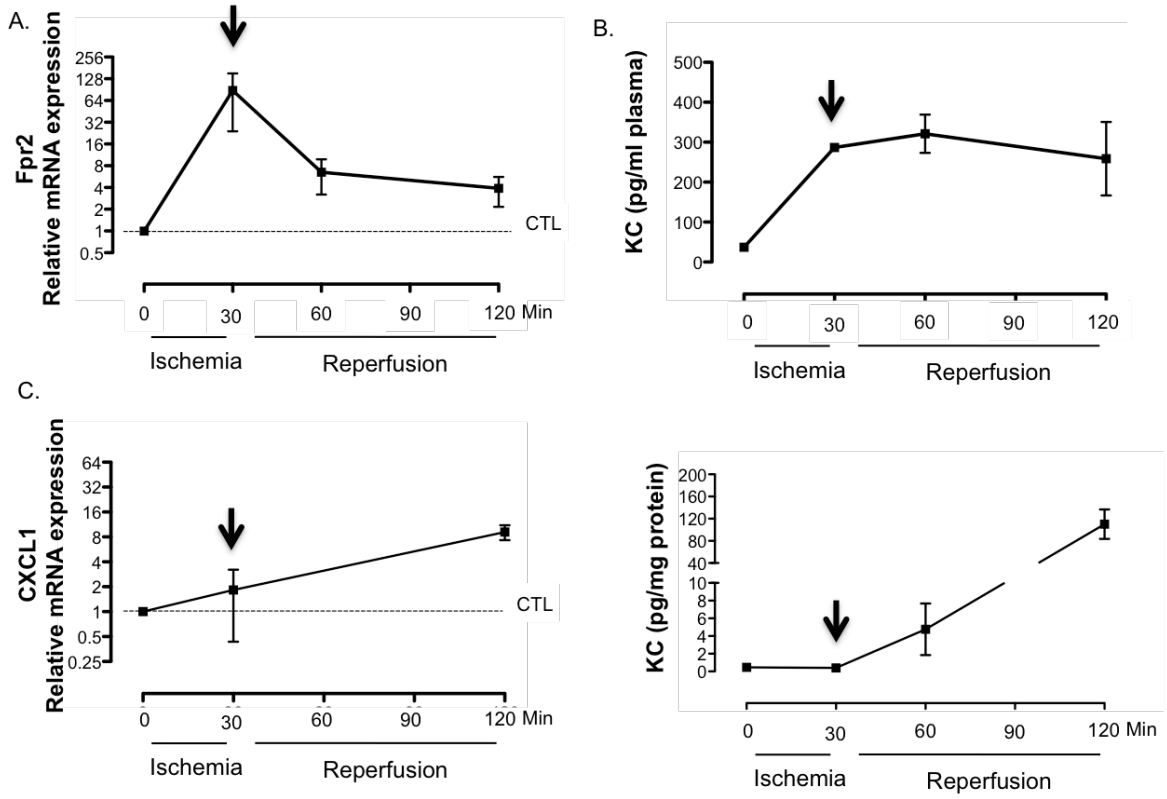


Figure 32: Fpr2 upregulation in the heart during ischemia is not connected with an increase in neutrophils.

AMI (30+90 min I/R) was induced in WT mice. At indicated time points hearts and plasma were collected and analysed by RT-PCR following the relative quantification method or ELISA. (A) Fpr2 – reused from figure 20 A for comparative purpose (C) CXCL1 mRNA modulation by AMI in the heart. KC induction in plasma (C) and in heart (D). The response produced in control mice (no AMI) was taken as 1 and data represent the upregulation (>1) or downregulation (<1) of mRNA expression. GAPDH was used as endogenous control. Data are showed as mean ± SEM of 8 to 15 mice per time point.

4.3.2 Effects of AMI in Fpr2/3 KO mice – Heart analyses

Hearts excised from WT and Fpr2/3 KO mice after acute myocardial infarct were studied and compared with the corresponding hearts of sham mice. The cardiac muscle of WT and Fpr2/3 KO mice, after AMI, showed an alteration of the classical structure as compared with the hearts of respective sham-operated mice. The classical cardiac morphology characterized by cross-striations formed by alternating segments of thick (myosin) and thin (actin) protein filaments (myofibril) was damaged (Figure 33).

In term of cell infiltration CXCL1/KC, CCL2/MCP1 and MPO levels were quantified. AMI induced an increase of the neutrophil chemoattractant chemokine CXCL1/KC (at both gene and protein level) in WT and Fpr2/3 KO mice ($p<0.001$ *vs.* corresponding sham). After AMI, CXCL1/KC levels were significant higher in Fpr2/3 KO mice when compared with WT mice ($p<0.05$) (Figure 34 A). The level of the MPO, expressed at high abundance in neutrophils (Figure 35), was also evaluated. No significant changes were observed between sham and AMI mice in both WT and Fpr2/3 KO mice (Figure 35). The AMI protocol induced an increase of CCL2 mRNA, with higher expression in the KO in comparison with WT mice ($p<0.01$). CCL2 is also referred to as monocyte chemotactic protein-1 (MCP1). CCL2/MCP1 recruits monocytes, memory T cells and dendritic cells to the sites of inflammation. No significant changes of the corresponding MCP1 protein were however observed (Figure 35 B).

Levels of pro- and anti-inflammatory cytokines and other chemokines were also measured (Table 2). Moreover, the pro-inflammatory protein serum amyloid A (SAA) was studied in view of its ability to activate Fpr2. TNFα, IL-6, IL-1β, IL-10 and SAA mRNA expression were upregulated after AMI in both WT and Fpr2/3 KO mice. TNFα mRNA upregulation was significantly higher in Fpr2/3 KO mice compared with WT mice ($p<0.05$). CCL5 mRNA expression was downregulated in WT mice and upregulated in KO mice ($p<0.001$). In terms of protein expression only Fpr2/3 KO mice showed an increase of TNFα and IL-6 after acute myocardial infarct ($p<0.05$) (Figure 36, 37).

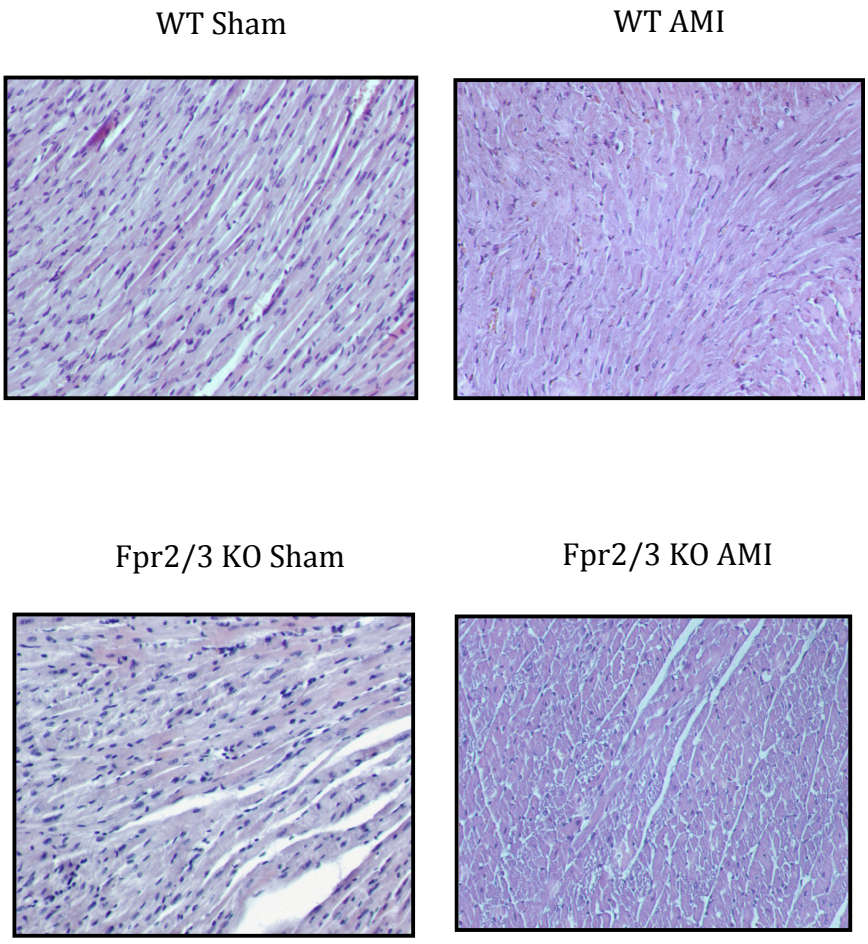


Figure 33: Morphology of Heart of WT and Fpr2/3 KO mice after AMI.
Representative images of H&E staining of heart removed from WT and Fpr2/3 KO mice, in sham condition or after 30 minutes of LADCA occlusion and 90 minutes of reperfusion. Pictures are representative of at least 6 different slices of heart harvested from 5 to 8 mice.

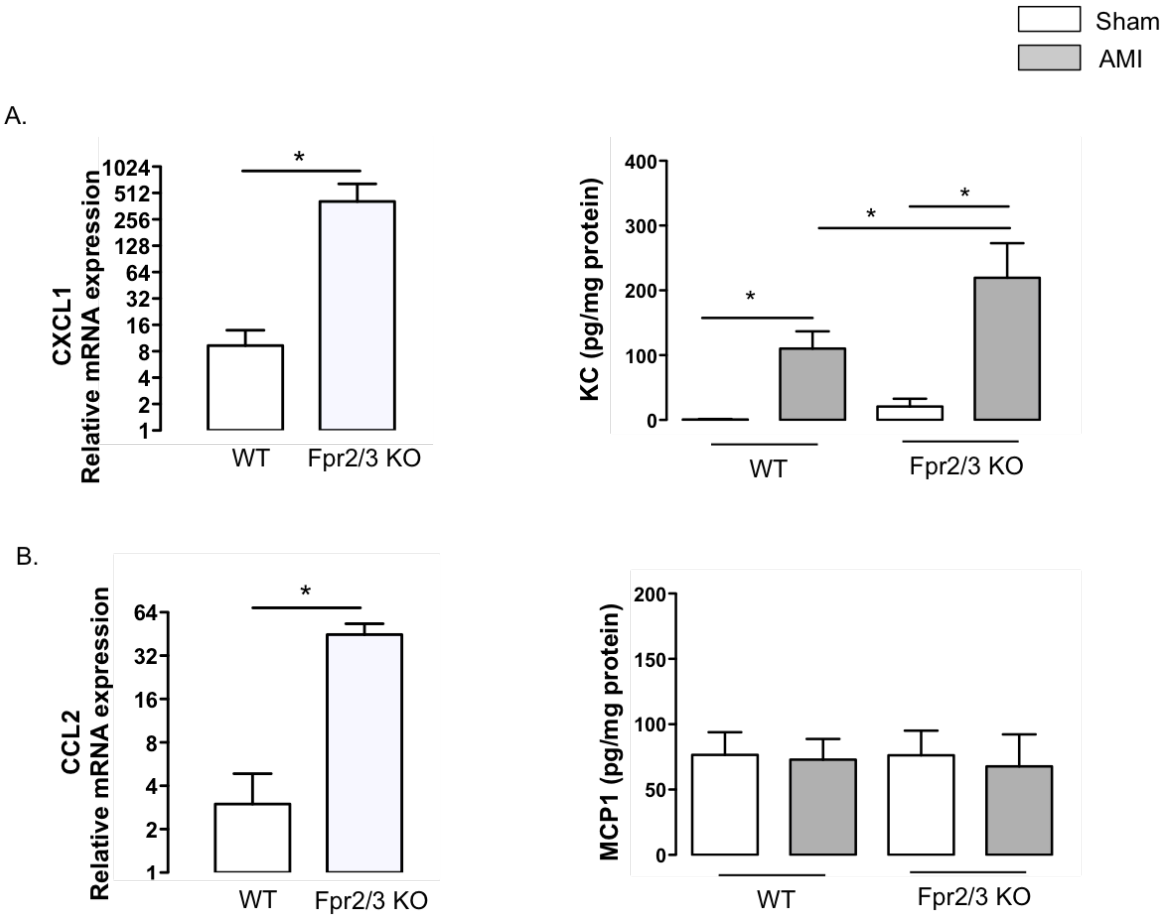
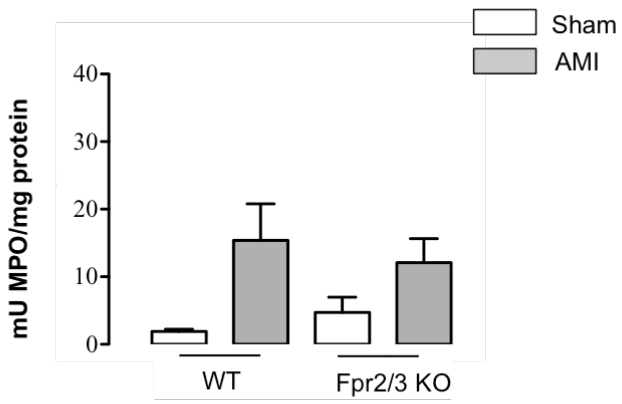


Figure 34: Analysis of cells infiltration in heart of WT and Fpr2/3 KO mice after AMI: CXCL1/KC and CCL2/MCP1 expression.

Genes and proteins expression of CXCL1/KC and CCL2/MCP1 in hearts of WT and Fpr2/3 KO mice subjected to 30 minutes of LADCA occlusion and 90 minutes of reperfusion. (A) AMI induced an increase of CXCL1 mRNA and KC protein ($p<0.001$ *vs.* sham mice) in WT and Fpr2/3 KO mice. After AMI, the upregulation of CXCL1 and KC levels was significant higher in Fpr2/3 KO mice compared with WT mice ($p<0.05$). (B) AMI up regulated the levels of CCL2 mRNA in both WT and Fpr2 KO mice with higher effect in the KO ($p<0.01$ *vs.* WT). No significant changes in MCP1 levels were detected. mRNA was quantified using the relative quantification method. Data were expressed as an upregulation (>1) or downregulation (<1) of mRNA compared to the levels in control mice (no AMI) taken as 1. GAPDH was used as endogenous control. Data are mean ± SEM of 8 to 15 mice per group.

C.



D.

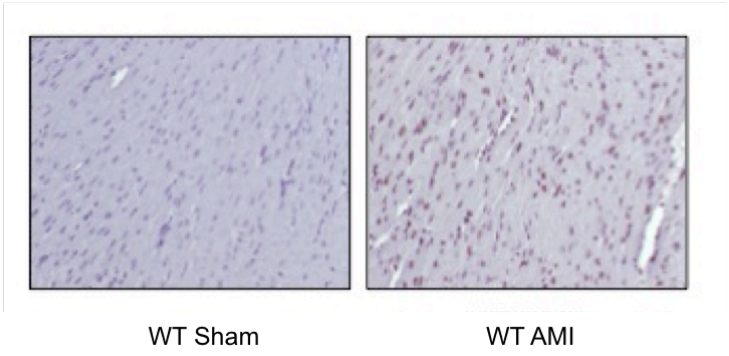


Figure 35: Analysis of cells infiltration in Heart of WT and Fpr2/3 KO mice after AMI: MPO levels.

Myeloperoxidase quantification and localization in hearts of WT and Fpr2/3 KO mice where the AMI procedure characterised by 30+90 min I/R of the heart was applied. (A) AMI induced a not significant increase in MPO levels in both WT and Fpr2/3 KO mice. (B) MPO was detected in fixed paraffin-embedded slides of WT and Fpr2/3 KO mice hearts in sham condition (left panel) or after AMI (right panel). The Anti-Myeloperoxidase antibody (Abcam, ab9535, 1:50) was used and representative pictures of at least 6 different slices of hearts harvested from 5 to 8 mice are shown. Data are showed as mean ± SEM of 8 to 15 mice per group.

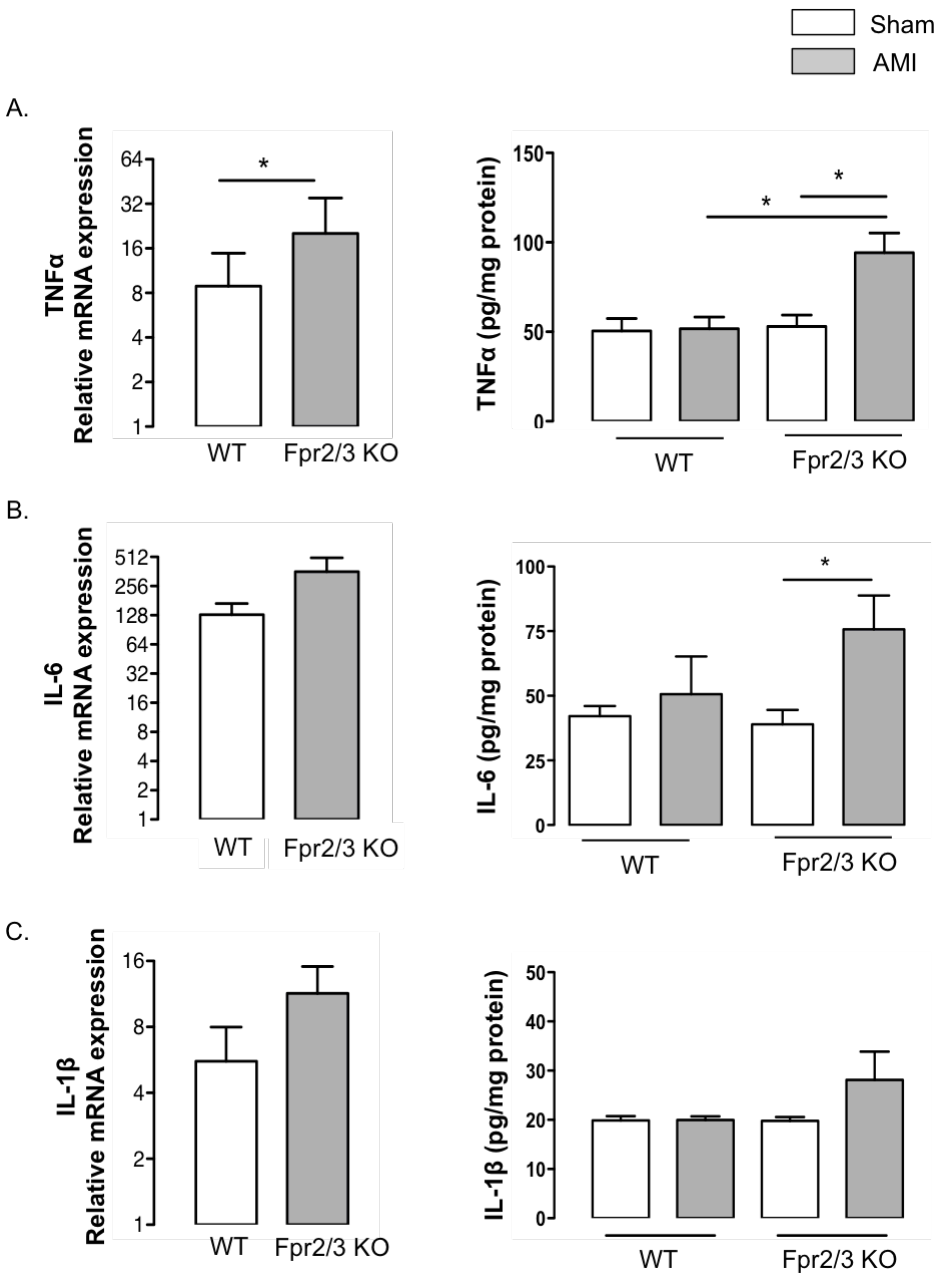


Figure 36: Screening of cytokines in hearts of WT and Fpr2/3 KO mice after AMI.
Levels of mRNAs (left panel) and proteins (right panel) of TNFα, IL-6 and IL-1β in hearts of WT and Fpr2/3 KO mice after acute myocardial infarct. (A) AMI upregulated TNFα mRNA in both WT and Fpr2/3 KO mice. The relative mRNA expression was higher in Fpr2/3 KO mice compared with WT mice ($p<0.05$). TNFα protein levels were upregulated by AMI in Fpr2/3 mice ($p<0.05$ *vs.* Fpr2/3 sham mice and WT AMI). (B) IL-6 protein expression was increased by AMI only in Frp2/3 KO mice ($p<0.05$). (C) No significant changes in IL-1β mRNA and protein expression were detected. Data are representative as mean ± SEM of 6 to 10 mice per group.

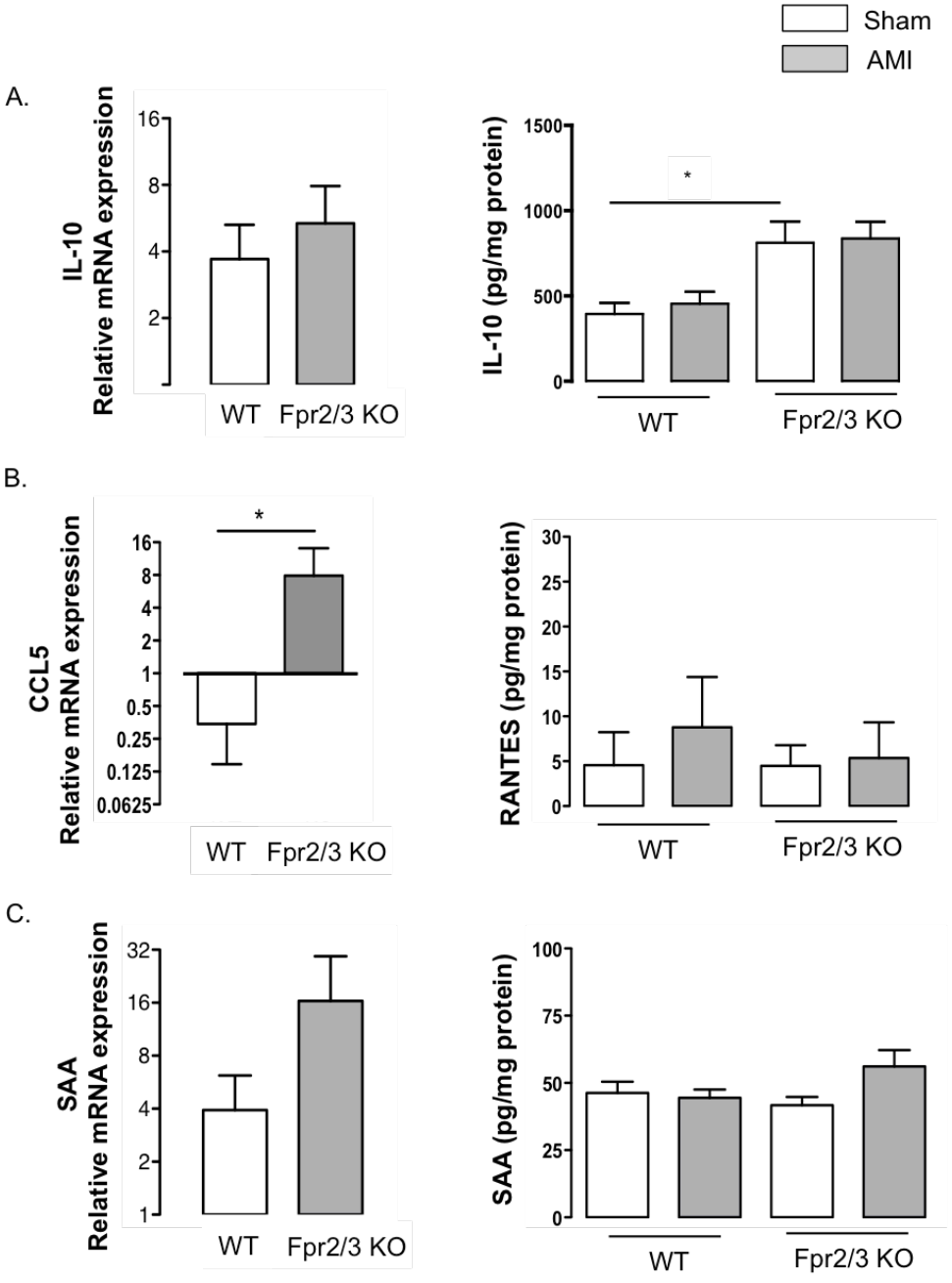


Figure 37: Screening of cytokines, chemokines and inflammatory mediators in hearts of WT and Fpr2/3 KO mice after AMI.

mRNAs (left panel) and proteins (right panel) quantification of IL-10, CCL5/RANTES and SAA in hearts of WT and Fpr2/3 KO mice after ischemia/reperfusion through LADCA occlusion.

(A) AMI increased the levels of IL-10 mRNA in both WT and Fpr2/3 KO mice. Fpr2/3 KO sham mice were characterized by higher expression of IL-10 compared with WT mice ($p<0.05$). (B) CCL5 mRNA was downregulated in WT mice and upregulated in KO mice ($p<0.001$ WT *vs.* Fpr2/3 KO) by AMI. No changes in protein expression were induced by LADCA occlusion. (C) No significant changes in SAA mRNA and protein levels were detected. Data are expressed as mean ± SEM of 6 to 10 mice per group.

Heart (pg/mg)					
		Sham *(Mean±SEM)*	**AMI** *(Mean±SEM)*	**P value** (Sham vs AMI)	**P value** (AMI: WT vs KO)
KC	*WT*	0.621±0.5	101.548±24.02	***p<0.01***	***p<0.05***
	Fpr2 KO	15.57±9.53	219.50±53.43	***p<0.001***	***KO: Higher CXCL1 mRna upregulation***
MCP1	*WT*	76.625±17.34	72.91±15.89	*p>0.05*	*p>0.05*
	Fpr2 KO	76.17±18.91	67.83±24.41	*p>0.05*	***KO: Higher CCL2 mRna upregulation***
TNFα	*WT*	31.731±3.66	49.02±6.23	*p>0.05*	***p<0.001***
	Fpr2 KO	53.015±6.42	94.19±11.07	***p<0.01***	***KO: Higher* TNFα *mRna upregulation***
IL-1β	*WT*	19.907±0.86	19.98±0.74	*p>0.05*	*p>0.05*
	Fpr2 KO	19.772±0.8	28.128±5.73	*p>0.05*	
IL-10	*WT*	392.68±65.24	453.35±70.26	*p>0.05*	*p>0.05*
	Fpr2 KO	811.89±123.41	863.008±97.24	*p>0.05*	

Table 2: Summary of chemokines and cytokines modulation induced by AMI in heart harvested from WT and Fpr2/3 KO mice.

Summary of proteins and mRna modulation of KC/CXCL1, MCP1/CCL2, TNFα, IL-1β and IL-10 in hearts of WT and Fpr2/3 KO mice after ischemia/reperfusion through LADCA occlusion.

4.2.4 Effects of AMI in Fpr2/3 KO mice – plasma analyses

At the end of the 90 minutes of reperfusion, plasma cytokine/chemokine profiles from WT and Fpr2/3 KO mice was also analysed. The AMI procedure upregulated CXCL1/KC levels in both WT ($p<0.001$) and Fpr2/3 KO mice ($p<0.05$). No significant changes in MCP1 were detected (Figure 38).

Many endogenous mediators are released into the plasma by cells that infiltrate the site of challenge, especially during ischemia reperfusion injury. Using cyclo-oxygenases or lipoxygenase, leukocytes rapidly biosynthesize mediators from membrane-derived arachidonic acid or its derivates. In order to study the levels of activation of inflammation, PUFAs (Polyunsaturated fatty acid, ω3 – ω6) metabolites derived from the cyclooxygenase (COX) and lipooxygenase (LOX) pathway and from auto oxidation were monitored during AMI (Table 3). COX is an enzyme responsible for the formation of biological mediators called prostanoids catalysing the conversion of free essential fatty acids to prostaglandins, prostacyclin and thromboxane. COX converts arachidonic acid (AA, a ω-6 PUFA) to prostaglandin H_2 (PGH_2), the precursor of the series-2 prostanoids. Here we analyzed different COX's metabolites (Table 3) and detected significant modulation of PGE_2, TXB_2, PGA_1 and PGI_2, with data showed in Figure 39.

Prostaglandin E synthase generates prostaglandin E_2 (PGE_2). PGE_2 is synthesized in inflammation where it acts as a vasodilator. Synergistically with other mediators such as histamine and bradykinin, PGE_2 is also able to cause an increase in vascular permeability and oedema. It is a central mediator of febrile response

triggered. AMI was able to induce PGE_2 only in Fpr2/3 KO mice ($p<0.01$ *vs.* KO Sham and $p<0.05$ *vs.* WT AMI) (Figure 39).

Thromboxane-A synthase generates thromboxane A_2 from PGH_2, and then this leads to downstream generation of the inactive metabolite/product TXB_2. TXA_2 is produced by activated platelets and possesses prothrombotic properties: it is involved in platelet activation and aggregation. Also in this case, only Fpr2/3 KO mice displayed increased TXA_2 levels both during ischemia ($p<0.01$) and at the end of AMI ($p<0.05$) (Figure 39).

The potent anti-inflammatory PGA_1, derived from dihomo-γ-linolenic acid 20:3 (DGLA), has been shown to cause renal vasodilation, stimulates renin release, inhibit NFκB activation and induce thermo-tolerance through modulation of heat shock protein 70. Fpr2/3 KO mice, after AMI, genereated less PGA_1 compared to WT mice ($p<0.05$) (Figure 39).

Prostacyclin is produced in endothelial cells from prostaglandin H_2 (PGH_2) by the action of the enzyme prostacyclin synthase. Prostacyclin I_2 (PGI_2) prevents formation of the platelet by inhibiting platelet activation. It is also vasodilator. In this case, Fpr2/3 KO mice had higher PGI_2, in ischemia ($p<0.05$) and at the end of reperfusion ($p<0.01$), when compared to sham mice (Figure 39).

Lipoxygenases are a family of iron-containing enzymes that catalyses the dioxygenation of PUFA in lipids containing a cis,cis-1,4-pentadiene structure. The metabolism of arachidonic acid (AA) and eicosapentaenoic acid (20:5(n-3), EPA)

through the lipoxygenase pathways leads to the generation of various biologically active eicosanoids like the proresolving mediator and FPR2/ALX agonist lipoxin A_4 (LXA_4) or the pro inflammatory leukotriene B_4 (LTB_4). LTB_4 induces adhesion and activation of leukocytes on the endothelium and induces the formation of reactive oxygen species. Fpr2/3 KO mice had a lack of LXA_4 after AMI ($p<0.05$). Both WT ($p<0.05$) and Fpr2/3 KO mice ($p<0.01$) had an increase of LTB_4 during ischemia but only WT mice showed a decrease in LTB_4 during reperfusion ($p<0.01$ KO AMI *vs.* KO Sham) (Figure 40). Also the pro-inflammatory 12-hydroxyeicosatetraenoic acid (12-HETE), a metabolite of AA produced by the enzyme 12-lipoxygenase (12-LOX), was significant increased only in Fpr2/3 KO both during ischemia ($p<0.05$) and at the end of reperfusion ($p<0.01$) (Figure 40). Analysing some of the intermediates of the lipoxygenase pathway such as the 5-hydroxyeicosatetraenoic acid (5-HETE) and 15-hydroxyeicosatetraenoic acid (15-HETE), we observed no changes for 5-HETE whereas an increase of 15-HETE was only detected in Fpr2/3 KO after ischemia ($p<0.05$; Figure 40).

Resolvins are lipid mediators synthesized from the essential omega 3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (22:6(n-3), DHA). They are induced in the resolution phase following acute inflammation. After AMI, Frp2/3 KO mice had higher levels of the resolvin D precursor 17-hydroxy docosahexaenoic Acid (17-HDoHE) ($p<0.05$) but less resolvin D_2 (RvD_2) ($p<0.05$) (Figure 41). In WT mice, AMI induced an increase of RvD_2 ($p<0.05$) (Figure 41). Only WT mice displayed an increase in the resolvin E precursor 18-HEPE after ischemia ($p<0.05$) (Figure 41).

The 14-hydroxy docosahexaenoic Acid (14-HDoHE), an autoxidation product of DHA, is a marker of oxidative stress. 14-HDoHE was produced by ischemia in both WT and Fpr2/3 KO mice ($p<0.05$). In Fpr2/3 KO mice the levels remained high even at the end of the reperfusion ($p<0.05$) (Figure 41).

A summary on the modulation of all mediators analysed during acute myocardial infarct can be recapitulated with the heat map of ω3 and ω6 metabolites as measured at the end of acute myocardial infarct (Figure 42).

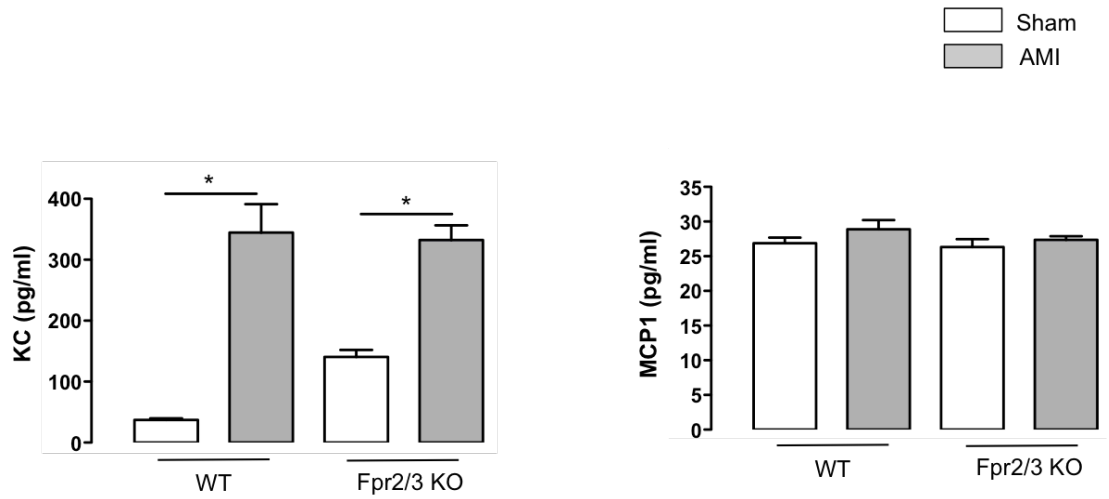


Figure 38: KC and MCP1 plasma levels in WT and Fpr2/3 KO mice after AMI.
Acute myocardial infarct procedure characterised by 30+90 min I/R of the heart was applied to WT and Fpr2/3 KO mice. At the end of reperfusion whole blood was collected by cardiac puncture and plasma obtained by centrifugation and analysed. AMI upregulated WT ($p<0.001$) and Fpr2/3 KO KC levels ($p<0.05$). No significant changes in MCP1 expression were detected. Data are expressed as mean ± SEM of 6 to 10 mice per group.

Precursors	Pathway	Metabolites
DGLA *dihomo-γ-linolenic acid 20:3 (ω−6)*	COX	PGA1
AA *Arachidonic acid 20:4 (ω-6)*	COX	6κPGF1α
		TxB2
		PGE2
		8-iso-PGA2
		15-d-PGJ2
	LOX	5 Oxo-ETE
		5-HETE
		15-HETE
		LxB4
		LxA4
		LtB4
		8-HETE
		12-HETE
	CYP	5, 6-EET
		8, 9-EET
		11, 12-EET
		14, 15-EET
		5, 6-DiHETE
EPA *eicosapentaenoic acid 20:5 (ω−3)*	COX	PGE3
	Autoxidation	18-HEPE
	LOX	LtB5
DHA *Dososahexaenoic acid 22:6(ω-3)*	Autoxidation LOX Epoxidation	17-HDoHE
		PDx
		RvD1
		RvD2
		7-MaR1
	Autoxidation	14-HDOHE

Table 3: Summary of the metabolites analysed in plasma of WT and Fpr2/3 KO.
Plasma of WT and Fpr2/3 KO mice were collected after ischemia or after reperfusion of the heart and analysed by liquid chromatography and mass spectrometry. Selected metabolites from the COX, LOX, Cyp pathways and from autooxidation were studied, as detailed here with indication of their precursors.

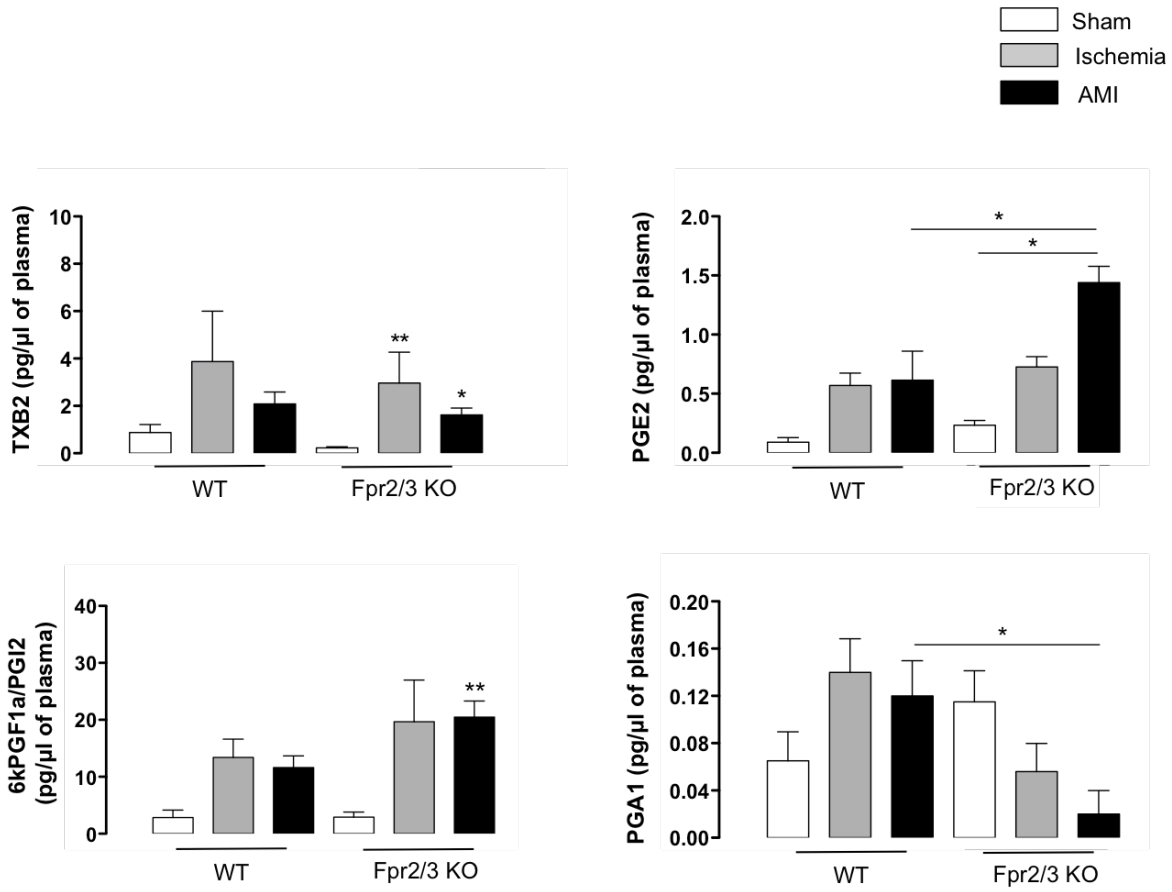


Figure 39: Plasma metabolites derived from the COX pathway.

Plasma collected from WT and Fpr2/3 KO mice in sham condition (white bar), at the end of ischemia (grey bar) or at the end of reperfusion (black bar) has been analysed. AMI was able to induce, only in Fpr2/3 KO mice, PGE2 ($p<0.01$ *vs.* KO Sham and $p<0.05$ *vs.* WT AMI) and TXA2. TXA2 was increased during ischemia ($p<0.01$) and at the end of AMI ($p<0.05$). Fpr2/3 KO mice, after AMI, had less PGA1 compared with WT mice ($p<0.05$) and higher PGI2, in ischemia ($p<0.05$) and at the end of reperfusion ($p<0.01$). Data are showed as mean ± SEM of 10 mice per group. (*$p<0.05$, **$p<0.01$ *vs.* respective sham or comparison indicates with connectors).

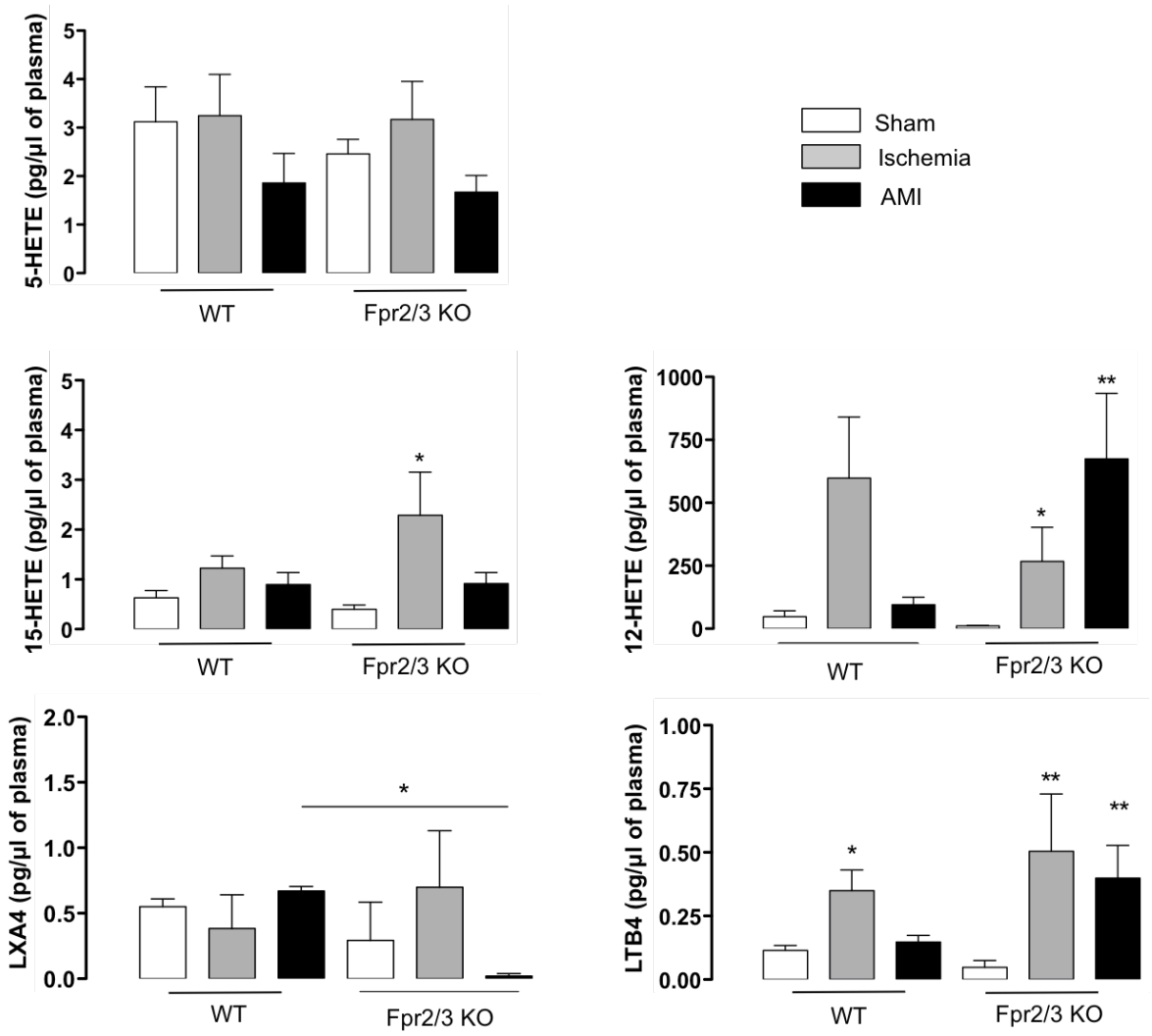


Figure 40: Plasma metabolites derived from the LOX pathway.

Plasma collected from WT and Fpr2/3 KO mice in sham condition (white bar), at the end of ischemia (grey bar) or at the end of reperfusion (black bar) has been studied. Fpr2/3 KO mice had a lack of LXA4 after AMI ($p<0.05$). LTB4 was increased during ischemia in both WT ($p<0.05$) and Fpr2/3 KO mice ($p<0.01$). In Fpr2/3 KO mice, LTB4 was significant higher also after AMI ($p<0.01$). Fpr2/3 KO mice had an increase of 12-HETE after ischemia ($p<0.05$) and at the end of reperfusion ($p<0.01$). 15-HETE increased in Fpr2/3 KO after ischemia ($p<0.05$). Data are expressed as mean ± SEM of 10 mice per group. (*$p<0.05$, **$p<0.01$ *vs.* respective sham or indicated comparison).

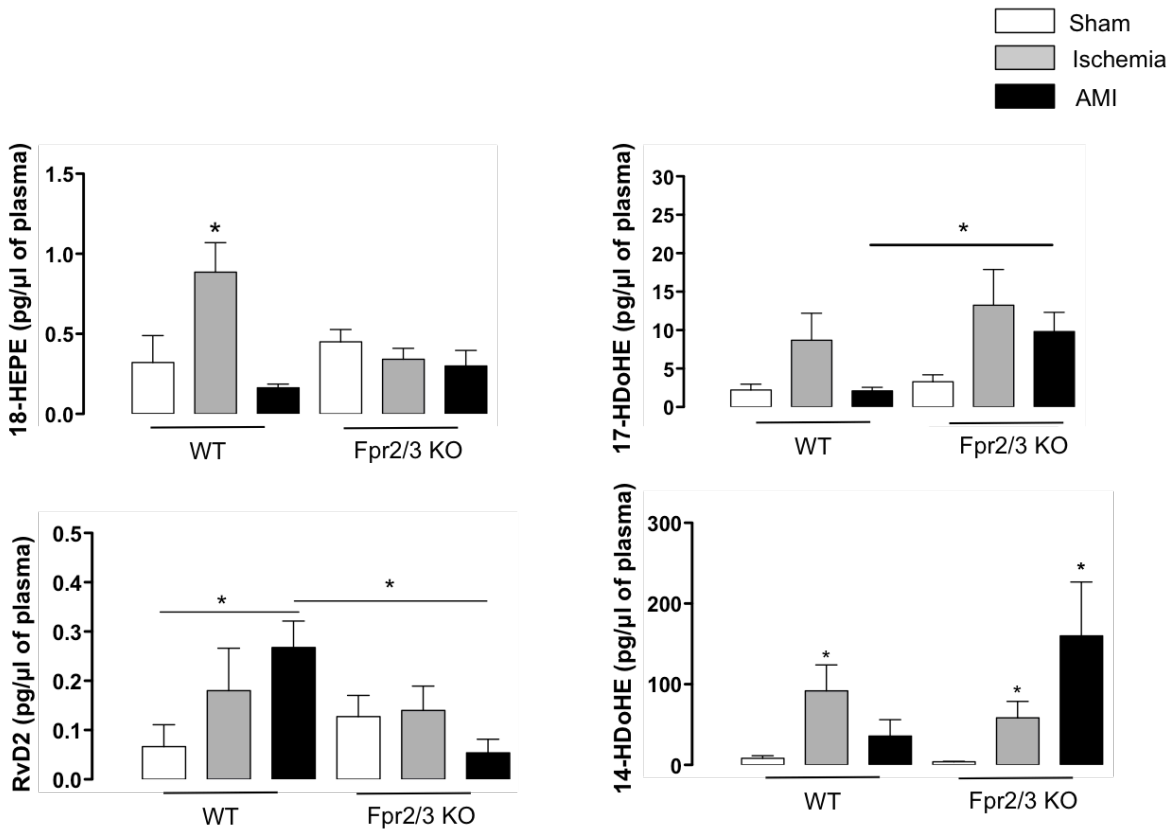


Figure 41: Plasma metabolites derived from the omega 3 pathways and from autoxidation. Plasma collected from WT and Fpr2/3 KO mice in sham condition (white bar), at the end of ischemia (grey bar) or at the end of reperfusion (black bar) has been analysed. After AMI, Frp2/3 KO mice had higher levels of 17-HDoHE ($p<0.05$) but less RvD2 ($p<0.05$). AMI induced RvD2 after ischemia and reperfusion ($p<0.05$) and 18-HEPE after ischemia ($p<0.05$) only in WT mice. 14-HDOHE was induced by ischemia in both WT and Fpr2/3 KO mice in ischemia ($p<0.05$) and only in Fpr2/3 KO mice at the end of the reperfusion ($p<0.05$). Data are showed as mean ± SEM of 10 mice per group. (*$p<0.05$, **$p<0.01$ *vs.* respective sham or indicated comparison).

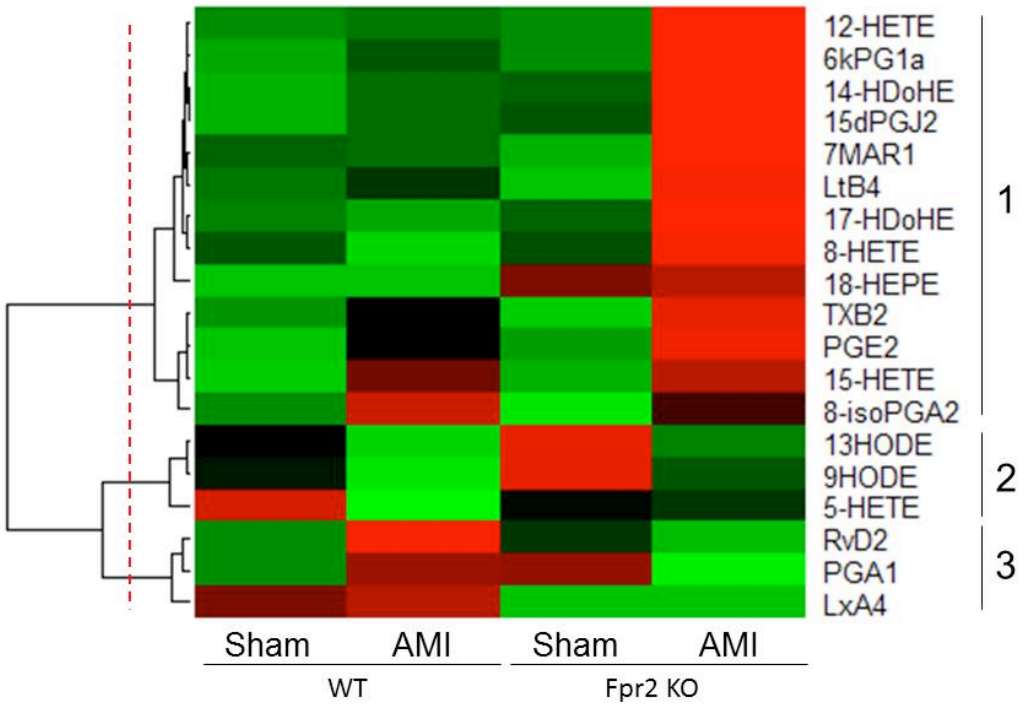


Figure 42: Heat map of ω3 and ω6 metabolites induced after AMI in WT and Fpr2/3 KO mice. Summary of metabolites modulated by AMI in the plasma of WT and Fpr2/3 KO mice at the end of ischemia and reperfusion (30 min + 90 min, respectively). Red bars represent higher levels of the metabolites (WT *vs.* KO). Changes to different intensity of green ending to black bars represent lower levels of the metabolites studied. Metabolites are divided in 3 groups:
1. Metabolites higher in Fpr2/3 KO *vs* Fpr2/3 KO sham mice or WT AMI mice, after AMI.
2. Metabolites decreased in Fpr2/3 KO mice after AMI in comparison to WT AMI mice.
3. Metabolites higher in WT AMI in comparison to Fpr2/3 KO mice post-AMI.

4.3 Role of Fpr2/3 in secondary organs injury after AMI

As reported in Chapter 3, Fpr2/3 are expressed in the lungs and in the kidneys. In order to study injury in secondary organ these tissues of WT and Fpr2/3 KO mice were removed at the end of acute myocardial infarct and examined.

4.3.1 Lung

The Fpr2 receptor is expressed in epithelial and endothelial cells of sham-operated WT mice (Figure 43 top panel). After acute myocardial infarct, Fpr2 is also expressed in infiltrated neutrophils (Figure 43 bottom panel). These representative pictures matched with biochemical data that showed that AMI was able to up-regulate lung mRNA expression of Fpr2 together with AnxA1 and Fpr1 mRNA (Figure 44 A). Both AnxA1 and Fpr1 mRNA were significantly higher in Fpr2/3 KO mice compared to WT mice ($p<0.001$) (Figure 44 A). AnxA1 and Fpr2, after AMI, were expressed and co-localized in the epithelial cells of the lung (Figure 44 B).

To evaluate tissue injury, the Koksel's protocol of lung injury evaluation was used (Ozdulger, Cinel et al. 2003, Koksel, Kaplan et al. 2005, Koksel, Ozdulger et al. 2005, Koksel, Yildirim et al. 2005, Koksel, Yildirim et al. 2005). Tissue oedema formation, infiltration of inflammatory cells and the integrity of the pulmonary architecture were evaluated. The injury was classified into four grades (Figure 45 A). After AMI, Fpr2/3 KO mice showed higher interstitial congestion, destruction of pulmonary structure and infiltration compared with WT mice ($p<0.01$) (Figure 45 B,C).

In terms of cell infiltration, the lungs of both WT and Fpr2/3 KO mice showed an increase in CXCL1/KC protein ($p<0.001$), however only WT mice had an increase of CXCL1/KC. Fpr2/3 KO mice showed decreased CXCL1/KC mRNA expression ($p<0.001$ *vs.* WT) (Figure 46 A). MPO expression significantly increased after AMI only in Fpr2/3 KO mice ($p<0.01$ *vs.* KO Sham, $p<0.05$ *vs.* WT AMI) (Figure 47). AMI was also able to increase CCL2 mRNA, with higher expression in KO tissue samples when compared to WT ones ($p<0.001$). No significant changes of MCP1 levels were observed in either strain of mouse (Figure 46 B). Also levels of CCL5/RANTES were quantified noting that only WT mice displayed an increase of RANTES after AMI ($p<0.01$ *vs.* CTL or KO AMI). In contrast, CCL5 mRNA expression was down regulated in both WT and Fpr2/3 KO mice ($p<0.01$; Figure 46 C).

Other cytokines and pro inflammatory molecules including TNFα, IL-6, IL-1β, IL-10 and SAA were also measured (Figure 48, 49). Only IL-6 and IL-10 protein levels were modulated by the ischemia/reperfusion injury of the heart. Specifically, Fpr2/3 KO mice had an increase of IL-6 ($p<0.05$) (Figure 48 B) whilst WT mice had an increase of IL-10 ($p<0.01$) (Figure 49 C) after AMI. In terms of mRNA expression, both WT and Fpr2/3 KO mice had an increase in IL-6 and IL-1β, with higher IL-1β mRNA levels in KO mice in comparison with WT ($p<0.001$) (Figure 48). Only WT mice had an increase in IL-10 mRNA ($p<0.05$) (Figure 49). No significant changes of TNFα and SAA were detected (Figure 48, 49).

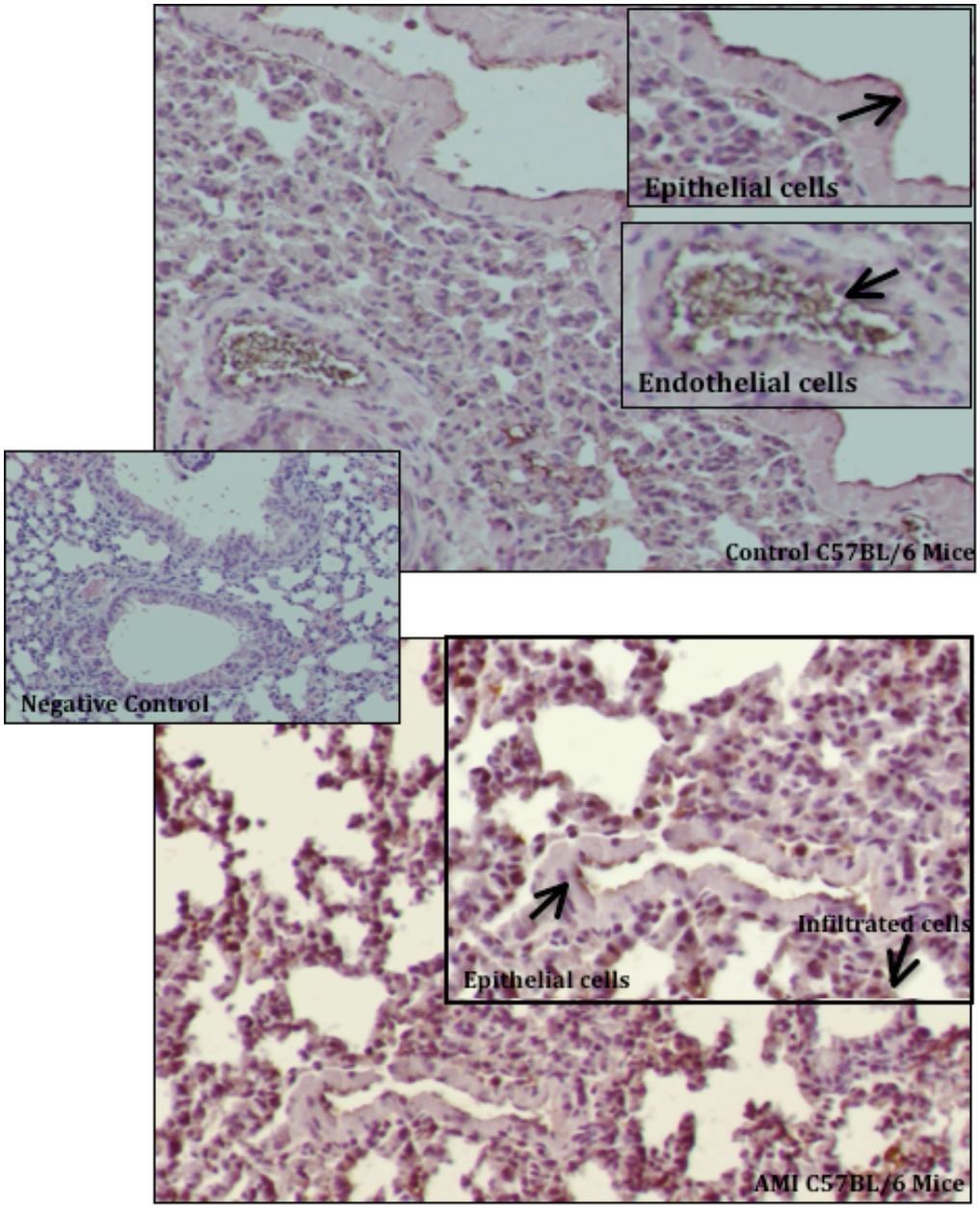
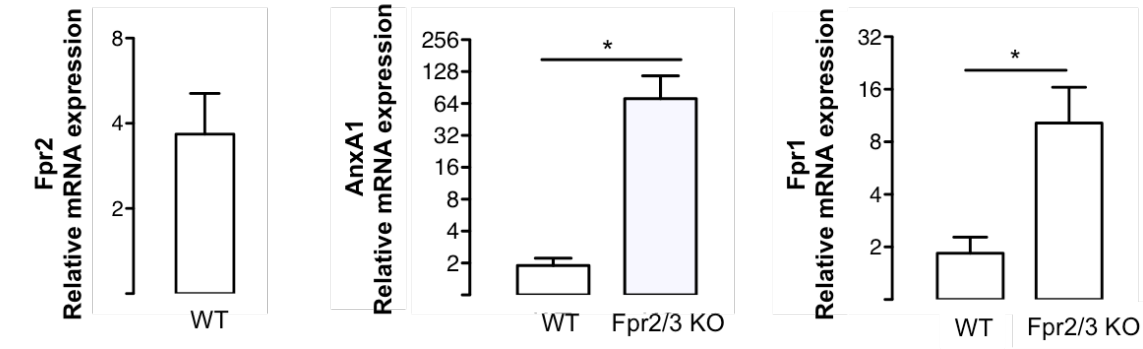


Figure 43: Fpr2 expression in lungs of sham or mice subjected to acute myocardial infarct. Fpr2 expression (brown staining) was detected in lungs fixed paraffin-embedded slides from WT and Fpr2/3 KO mice lungs in sham condition (top panel) or after AMI (bottom panel). Slides were stained with the anti-Fpr2 antibody (1:100). A negative control is shown. Pictures are representative of at least 6 different slices of hearts harvested from 6 to 10 mice.

A.



B.

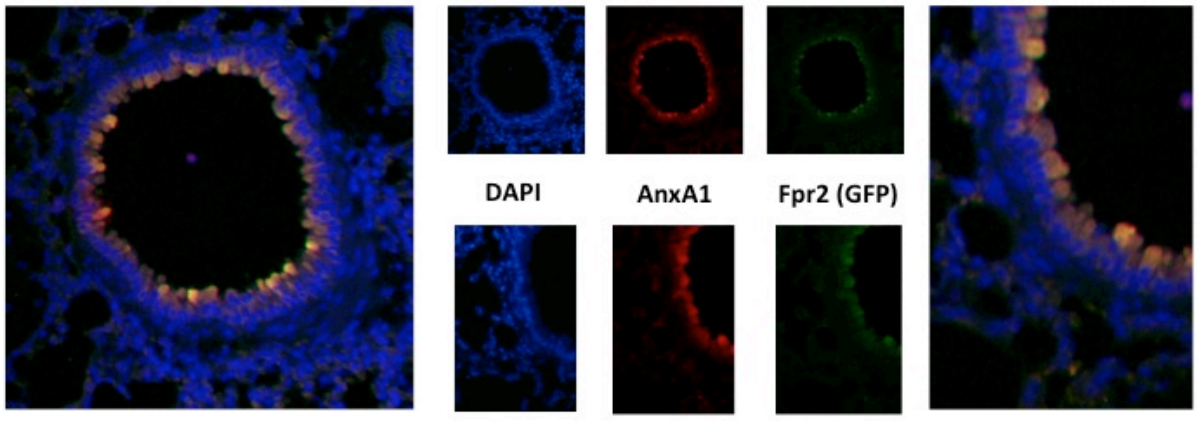


Figure 44: AnxA1, Fpr1 and Fpr2 expression in Lung of WT and Fpr2/3 KO mice after AMI. WT and Fpr2/3 KO mice were subjected to 30 min of LADCA occlusion and 90 min of reperfusion. Lungs were then excised and analysed by RT-PCR for AnxA1, Fpr1 and Fpr2 mRNA expression and by IHC for AnxA1 and Fpr2 localization. (A) Upregulation of Fpr2, AnxA1 and Fpr1 mRna expression in WT and Fpr2/3 KO mice after AMI with higher levels of AnxA1 and Fpr1 mRna in Fpr2/3 KO mice in comparison with WT ($p<0.001$). The response produced in control mice (no AMI) was considerated as 1 and the data were expressed as an upregulation (>1) or downregulation (<1) of mRNA expression after AMI. GAPDH was used as endogenous control. (B) Co-localization of AnxA1 (Red) and Fpr2 (Green-GFP) in epithelial cells of the lung of Fpr2/3 KO mice after I/R of the heart. Fixed paraffin-embedded slides were stained with DAPI (Blue) and AnxA1 antibody (1:1000). Fpr2 expression was detected thought the GFP fluorescence in the FL-1 channel. Images are representative of at least 6 different slices of lungs harvested from 5 or more mice. Data are showed as mean ± SEM of 8 to 15 mice per group

GRADE 1	Normal pulmunary histology
GRADE 2	Mild neutrophil leukocyte infiltrations and mild to moderate interstitial congestion
GRADE 3	Moderate neutrophil leukocyte infiltration, perivascular oedema formation and partial destruction of pulmonary architecture
GRADE 4	Dense neutrophils leukocyte infiltration, abscess formation and complete destruction of pulmonary architecture

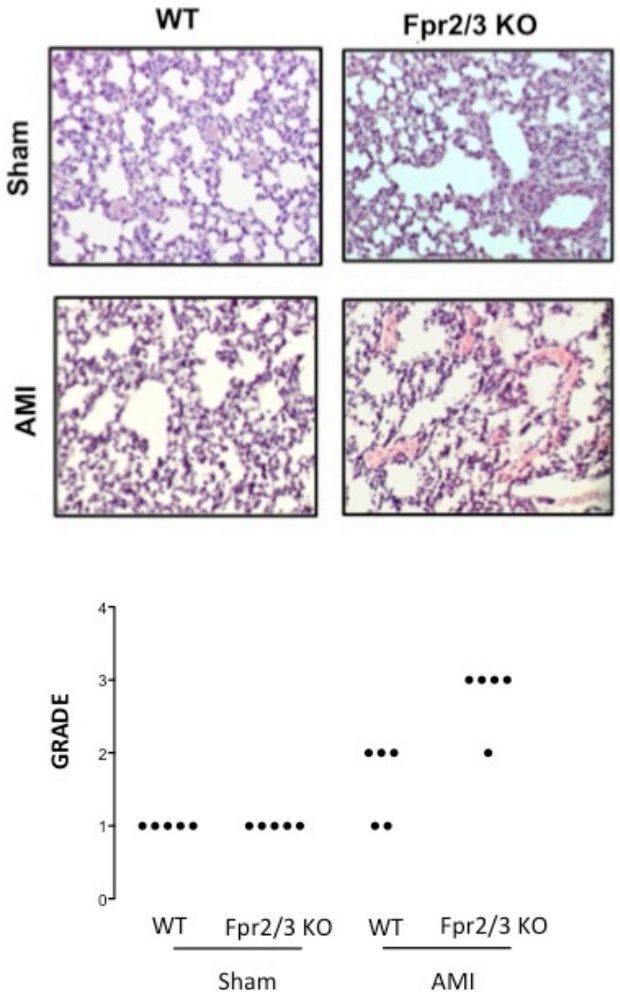


Figure 45: Lung injury evaluation of WT and Fpr2/3 KO mice after acute myocardial infarct. 30 minutes of LADCA occlusion followed by 90 minutes of reperfusion were applied to WT and Fpr2/3 KO mice. At the end of reperfusion lungs were collected, fixed in PFA and paraffin-embedded. Haematoxylin and eosin were used to stain the slides (pictures representative of 6 different slices of lungs harvested from 5 mice are shown) and the staining analysed following the grades of the Koksel's protocol of lung injury evaluation. Fpr2/3 KO mice showed higher lungs damage, after AMI, in comparison with WT mice ($p<0.01$). Data are mean ± SEM of 5 mice per group.

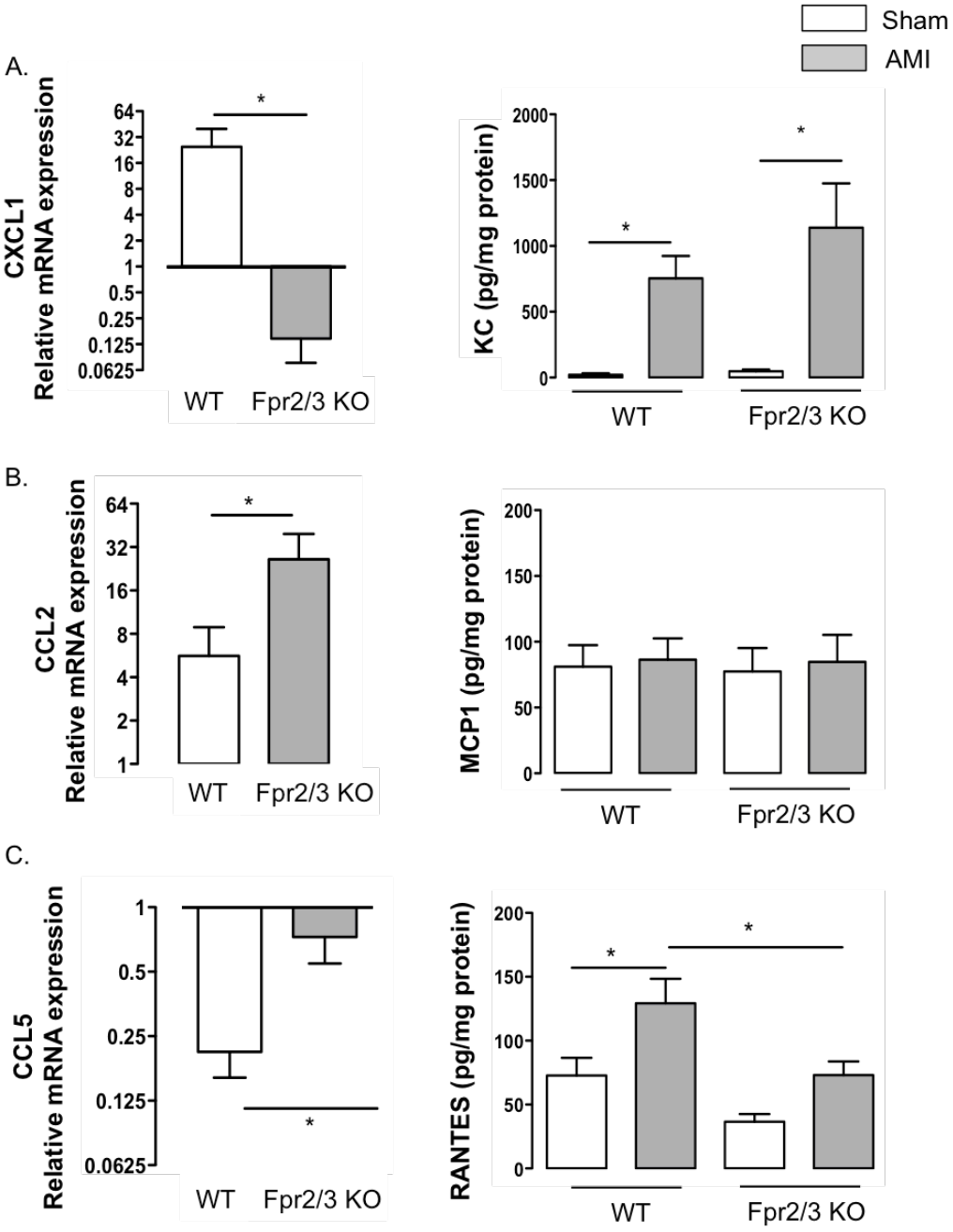


Figure 46: Analysis of cells infiltration in lungs of WT and Fpr2/3 KO mice after AMI.
Genes and proteins expression of CXCL1/KC, CCL2/MCP1 and CCL5/RANTES in lungs of WT and Fpr2/3 KO mice subjected to 30 min of ischemia and 90 min of reperfusion of the heart. (A) CXCL1 mRNA was up regulated in WT mice and down regulated in Fpr2/3 KO mice ($p<0.001$). KC was increased, by AMI, in both WT and Fpr2/3 KO mice ($p<0.001$). (B) AMI induced CCL2 mRNA, with higher values in KO mice compared with WT ($p<0.001$). No changes in MCP1 levels were detected (C) CCL5 mRna expression was down regulated in both WT and Fpr2/3 KO mice ($p<0.01$ WT *vs.* KO). WT mice had an increase of RANTES after AMI ($p<0.01$ *vs.* CTL or KO AMI). Following the relative quantification method the response induced in control mice (sham mice) was taken as 1. AMI data were expressed as upregulation (>1) or downregulation (<1) compared with sham mice. GAPDH was used as endogenous control. Data are showed as mean ± SEM of 6 to 10 mice per group.

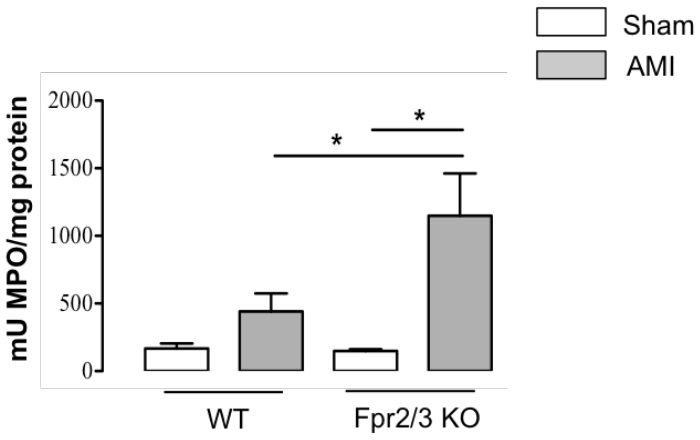


Figure 47: Analysis of cells infiltration of lungs of WT and Fpr2 KO mice after AMI: MPO levels.

30 min of ischemia of the heart, through LADCA occlusion, followed by 90 min of reperfusion were induced in WT and Fpr2/3 KO mice. At the end of reperfusion, lungs where excised and MPO levels analysed. AMI induced a significant increase in MPO only in Fpr2/3 KO mice ($p<0.01$ *vs.* KO Sham, $p<0.05$ *vs*. WT AMI). Data are showed as mean ± SEM of 8 to 10 mice per group.

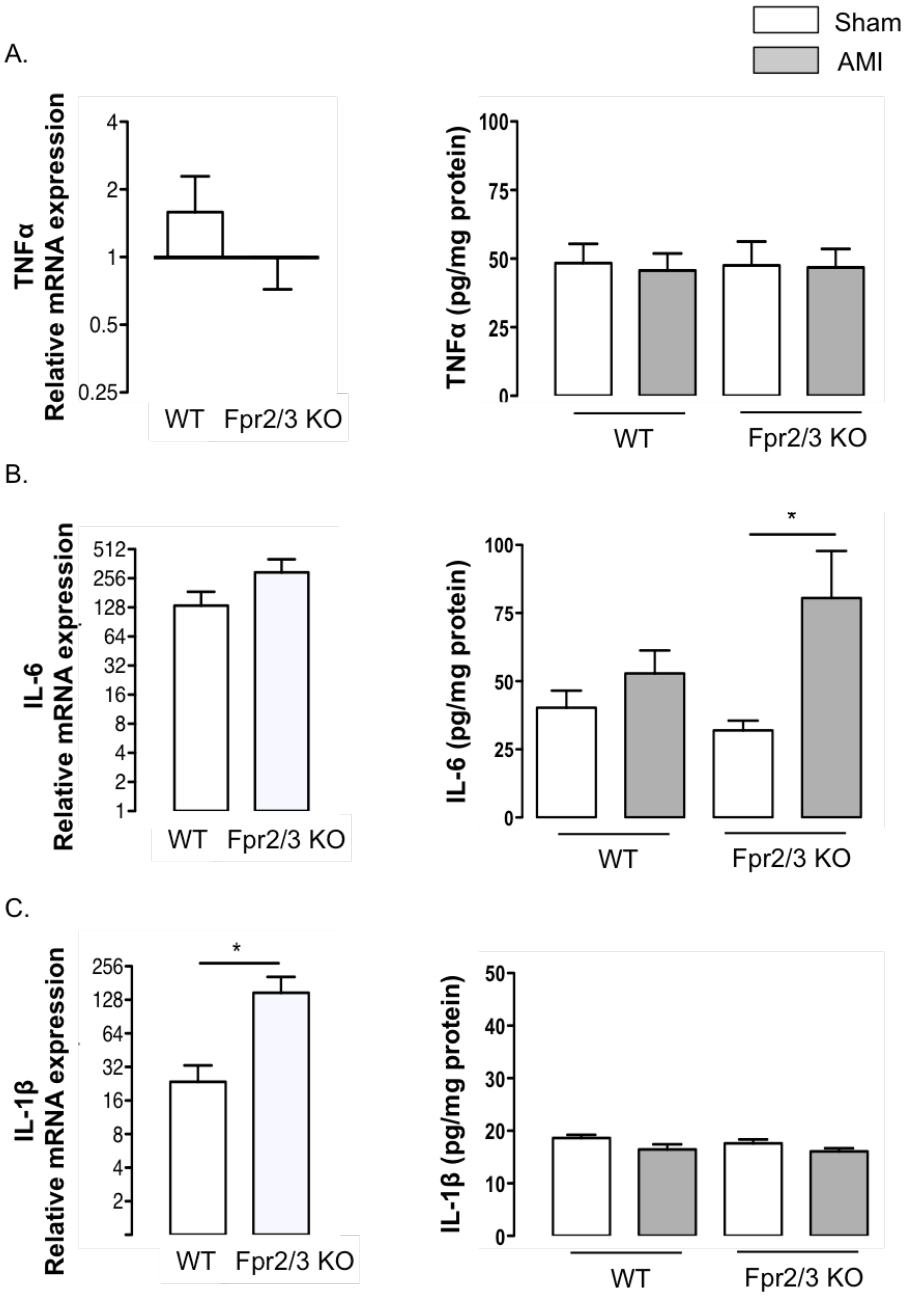


Figure 48: Screening of inflammatory mediators in lungs of WT and Fpr2/3 KO mice after AMI: TNFα, IL-6 and IL-1β.

Levels of gene (left panel) and proteins (right panel) expression of TNFα, IL-6 and IL-1β in lungs of WT and Fpr2/3 KO mice after acute myocardial infarct (30+90 min I/R). (A) No significant changes in TNFα were detected. (B,C) Both WT and Fpr2/3 KO mice had an increase in IL-6 and IL-1β mRNA, with higher levels of IL-1β in KO mice in comparison with WT ($p<0.001$). Fpr2/3 KO mice had also an increase of IL-6 after AMI ($p<0.05$). mRNA data are expressed as upregulation (>1) or downregulation (<1) of sham mice taken as 1, following the relative quantification method. GAPDH was used as endogenous control. Data are showed as mean ± SEM of 6 to 10 mice per group.

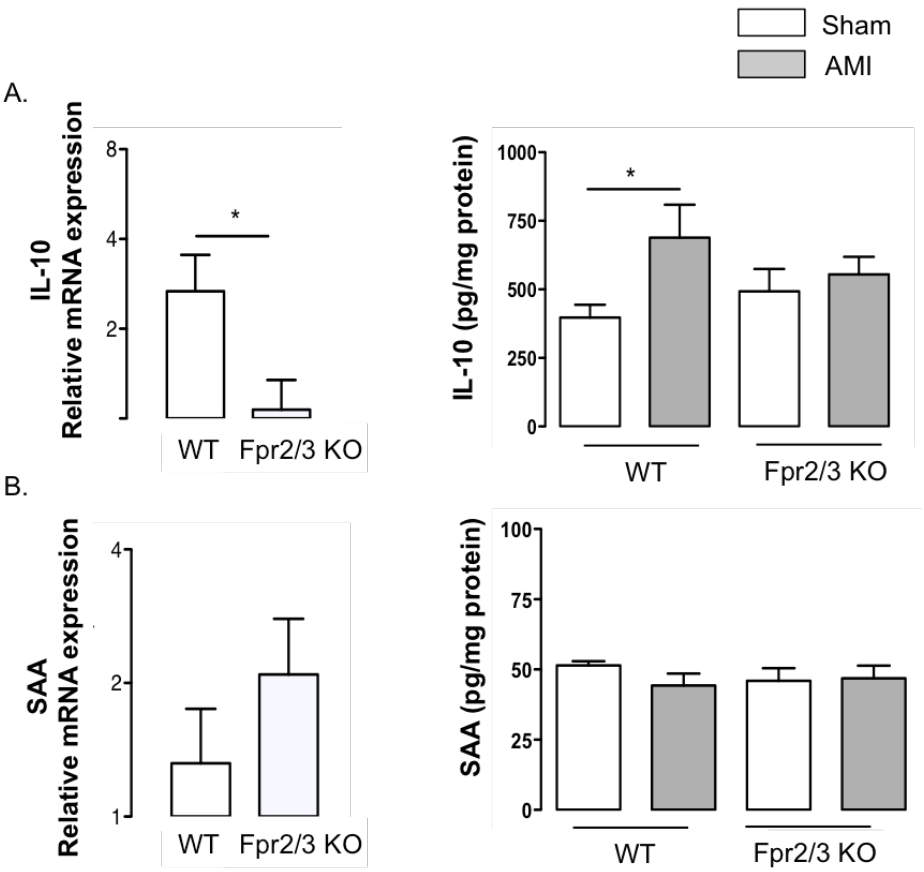


Figure 49: Screening of inflammatory mediators in Lungs of WT and Fpr2/3 KO mice after AMI: IL-10 and SAA.

Levels of gene (left panel) and proteins (right panel) expression of IL-10 and SAA in lungs of WT and Fpr2 KO mice after 30+90 min I/R of the heart through LADCA occlusion. (A) IL-10 was upregulated in WT mice by AMI both in terms of mRNA ($p<0.05$ *vs.* Fpr2/3 KO) and protein expression ($p<0.01$ *vs.* WT sham) (B) No significant changes in SAA levels were observed. Following the relative quantification method, mRNA data are expressed as upregulation (>1) or downregulation (<1) of sham mice taken as 1. GAPDH was used as endogenous control. Data are showed as mean ± SEM of 6 to 10 mice per group.

4.3.2 Kidney

As another organ prone to secondary injury, we examined the kidney of WT and Fpr2/3 KO mice following sham operation or acute myocardial infarct. AMI was able to up-regulate kidney expression of Fpr2 as seen by immunohistochemistry (Figure 50) and by quantifying mRNA expression (Figure 51). AMI also upregulated AnxA1 and Fpr1 mRNA. Fpr1 mRNA was significantly higher in Fpr2/3 KO mice in comparison to WT mice ($p<0.001$) (Figure 51). In terms of cytokines/chemokines, kidneys of Fpr2/3 KO mice contained increased levels of KC after AMI ($p<0.05$ *vs.* KO Sham, $p<0.01$ *vs.* WT AMI) (Figure 52 A). In contrast, kidneys harvested from WT mice had an increase in RANTES ($p<0.05$ *vs.* WT Sham, $p<0.01$ *vs.* KO AMI) (Figure 52 C). No significant changes in MCP1 (Figure 52 B) and MPO (Figure 52 A) expression were induced, with AMI, in both WT and Fpr2/3 KO mice.

After AMI, kidney samples of WT and Fpr2/3 KO mice displayed higher levels of IL-6 (Figure 53 A) and SAA (Figure 54 B) mRNA, with more pronounced values of IL-6 mRNA in the Fpr2/3 KO compared to WT mice ($p<0.001$). IL-1β mRNA was upregulated in both genotypes ($p<0.05$ WT *vs.* KO) (Figure 53 C). TNFα (Figure 53 A) and IL-10 (Figure 54 A) mRNA were down regulated in WT mice and upregulated in Fpr2/3 KO mice ($p<0.01$). In terms of protein expression only IL-10 was upregulated by AMI in Fpr2/3 KO mice ($p<0.05$ *vs.* KO SHAM and WT AMI) (Figure 54 A).

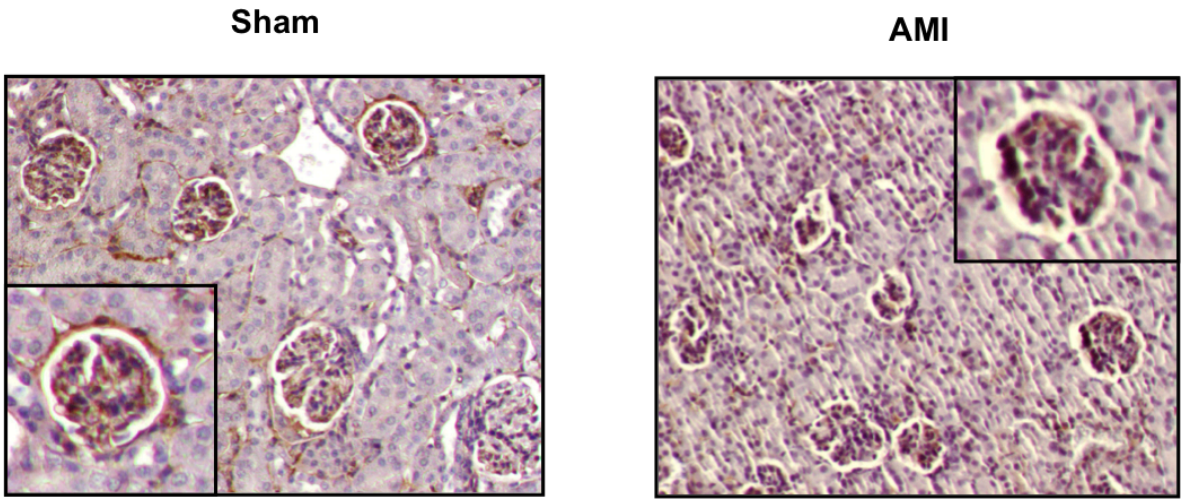


Figure 50: Fpr2 expression in kidneys of WT mice subjected to acute myocardial infarct. Fpr2 staining (1:200) on fixed paraffin-embedded kidneys harvested from WT mice after sham operation (left panel) or acute myocardial infarct (30+90 I/R of the heart) (right panel). Images are representative of at least 6 different slices of kidney from 5 to 10 mice.

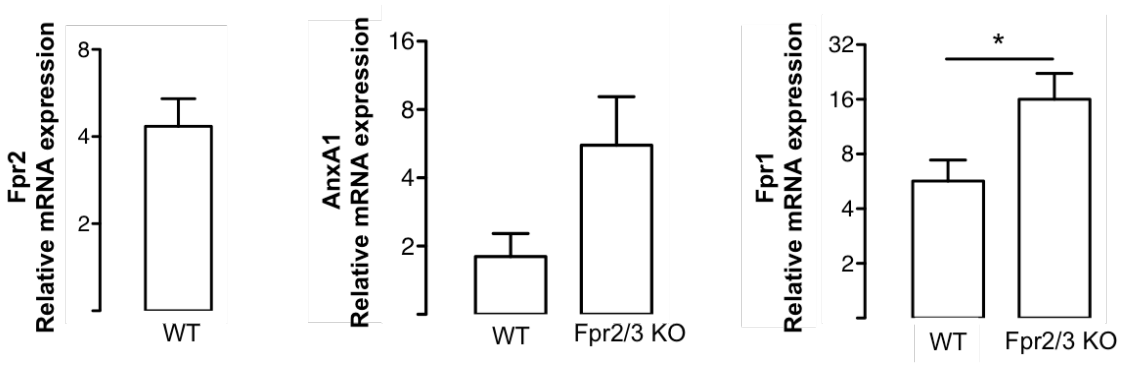


Figure 51: AnxA1, Fpr1 and Fpr2 expression in kidneys of WT and Fpr2/3 KO mice after AMI. mRNA analysis of kidney excised from WT and Fpr2/3 KO mice after 30 min of LADCA occlusion and 90 min of reperfusion using the relative quantification method. Fpr2, AnxA1 and Fpr1 mRNA were upregulated by AMI. Fpr1 mRNA was significantly higher in Fpr2/3 KO mice compare with WT mice ($p<0.001$). The response induced in control mice (no AMI) was taken as 1 and the AMI data expressed as upregulation (>1) or downregulation (<1) of mRNA expression. GAPDH was used as endogenous control. Data are showed as mean ± SEM of 6 to 10 mice per group.

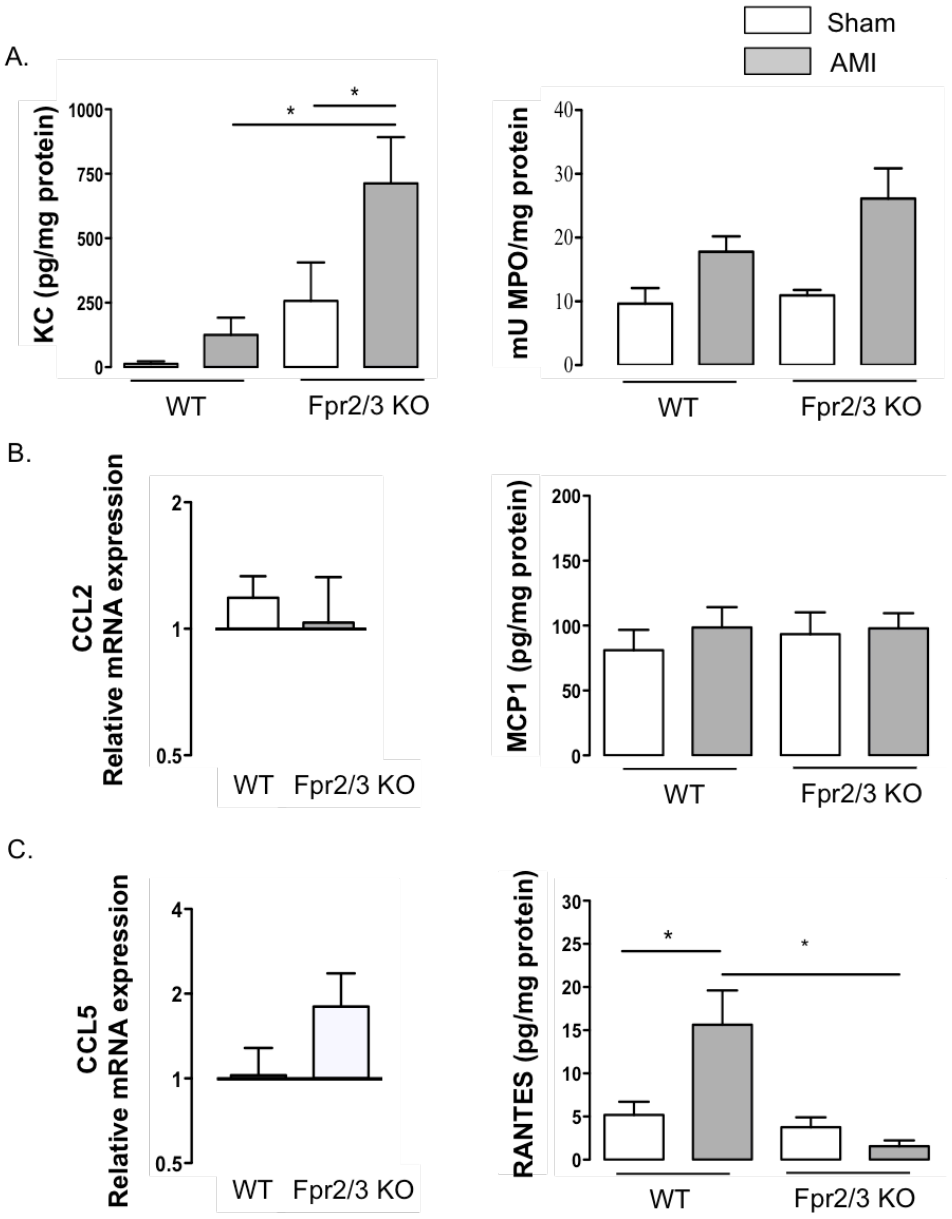


Figure 52: **Cells infiltration in kidneys of WT and Fpr2/3 KO mice after AMI.**
Kindneys of WT and Fpr2/3 KO mice were subjected to acute myocardial infarct (30+90 min I/R) were analysed by RT-PCR for mRNA expression and ELISA for protein expression. (A) Fpr2/3 KO mice, and not WT mice, had an increase of KC levels after AMI ($p<0.05$ *vs.* KO Sham, $p<0.01$ *vs.* WT AMI). No changes in MPO expression were observed. (B) No significant changes in CCL2/MCP1 were detected. (C) Kidneys of WT mice had an increase in RANTES ($p<0.05$ *vs.* WT Sham, $p<0.01$ *vs.* KO AMI) No changes in CCL5 mRNA were detected. mRNA data are expressed as upregulation (>1) or downregulation (<1) of sham mice taken as 1, following the relative quantification method. GAPDH was used as endogenous control. Data are showed as mean ± SEM of 6 to 10 mice per group

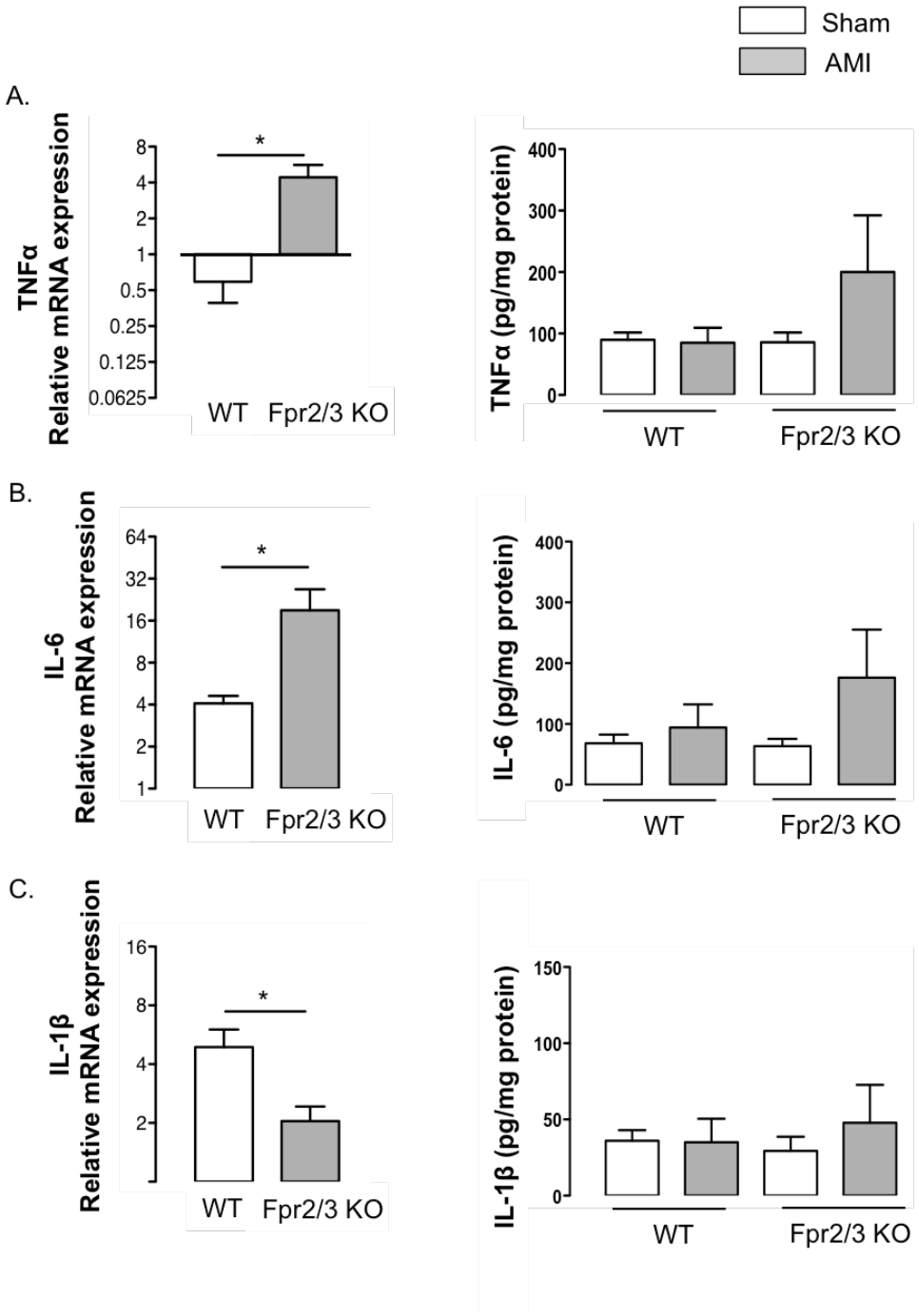


Figure 53: Inflammatory mediators in kidneys of WT and Fpr2/3 KO mice after AMI: TNFα, IL-6 and IL-1β.

WT and Fpr2/3 KO mice were subjected to sham or myocardial infarct surgery (30+90 min I/R of the heart). At the end of reperfusion kidneys were collected and analysed by RT-PCR, using the relative quantification method, for mRNA analyses and by ELISA for protein expression. (A) TNFα mRNA was down regulated in WT mice and upregulated in Fpr2/3 KO mice ($p<0.01$). (B, C) IL-6 and IL-1β mRNA were increased by AMI in both WT and Fpr2/3 KO mice. IL-6 mRNA was higher in the Fpr2/3 KO mice ($p<0.001$). IL-1β mRNA was higher in WT mice ($p<0.05$). No significant changes in protein expression were detected for all TNFα, IL-6 and IL-1β. mRNA data are compared with sham mice (taken as 1) and expressed as upregulation (>1) or downregulation (<1) of mRNA. GAPDH was used as endogenous control. Data are mean ± SEM of 6 to 10 mice per group.

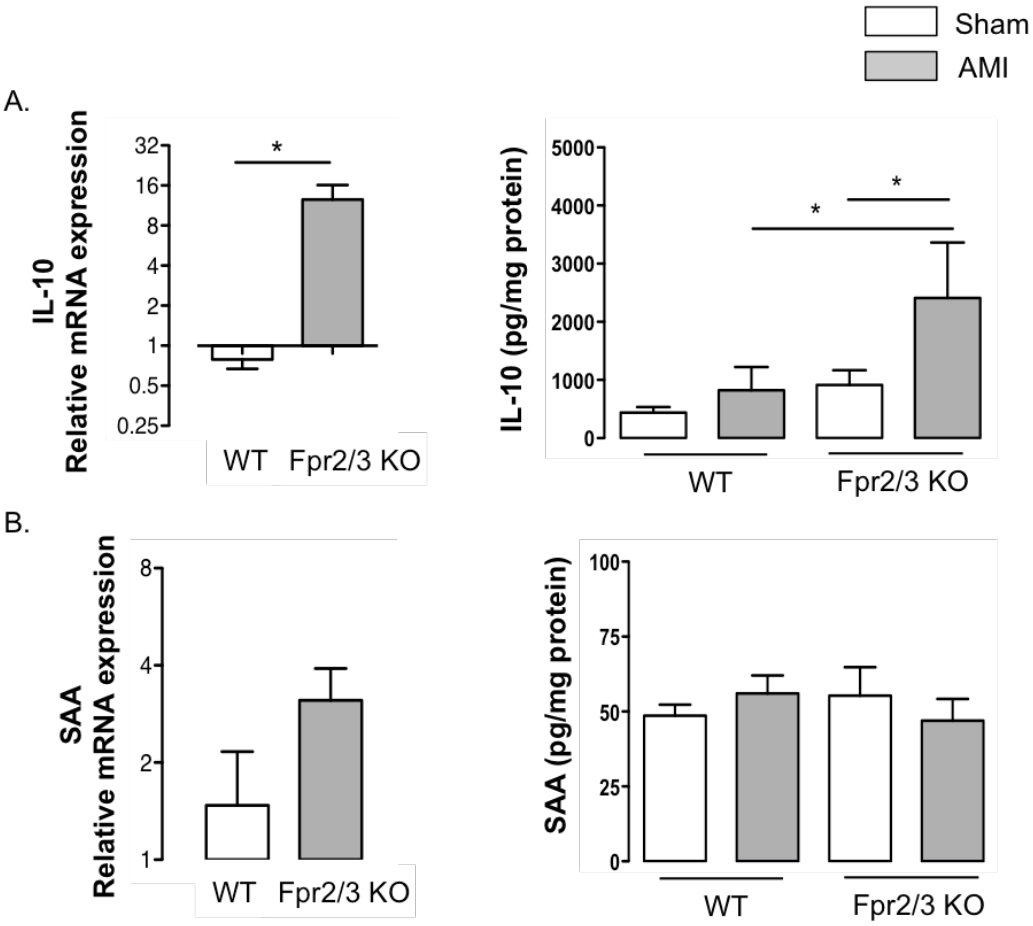


Figure 54: Cytokines and inflammatory mediators in kidneys of WT and Fpr2/3 KO mice after AMI: IL-10 and SAA.

Kidneys of WT and Fpr2/3 KO mice where the AMI procedure (30min of LADCA occlusion and 90 min of reperfusion) was induced were analysed for mRNA and protein expression of IL-10 and SAA. (A) IL-10 mRNA was down regulated in WT mice and upregulated in Fpr2/3 KO mice ($p<0.01$). The protein IL-10 was upregulated by AMI in Fpr2/3 KO mice ($p<0.05$ *vs.* KO SHAM and WT AMI). (B) SAA mRNA was upregulated by AMI in both WT and Fpr2/3 KO mice. No significant changes in protein levels were observed. Using the relative quantification method, mRNA data are showed as upregulation (>1) or downregulation (<1) of sham mice, taken as 1. GAPDH was used as endogenous control. Data are showed as mean ± SEM of 6 to 10 mice per group.

4.4 Activation of the IL-6 and JAK/STAT pathway by AnxA1 and Fpr2/3

Acute myocardial infarct induced an increase of IL-6 in plasma of both WT ($p<0.001$) and Fpr2/3 KO ($p<0.001$) and only in heart of the KO mice ($p<0.05$) (Figure 55). Several studies show how IL-6, in acute myocardial infarct, is able to activate the JAK/STAT3 pathway. The IL-6/JAK/STAT3 pathway elicits cardioprotection in the heart (Negoro, Kunisada et al. 2000). This fact may at a first sight suggests existence of an apparent contrast with the higher levels of IL-6 measured in Fpr2/3 KO mice which were concomitant to higher infarct size and heart damage markers. However, and in agreement with these differences, when we tested Fpr2/3 KO mice, we were unable to measure activation of the STAT3 ($p<0.001$ *vs*. WT AMI) (Figure 56).

JAK2 kinase plays an essential role in the cross-talk of the JAK/STAT pathway (Argetsinger, Campbell et al. 1993). AG-490, a tyrosine kinase inhibitor which inhibits protein tyrosine kinases by binding to the substrate binding site, is a potent and selective inhibitor of JAK2 kinase, with minimal or no effect on the kinase activity of other protein tyrosine kinases (Meydan, Grunberger et al. 1996). For this reason AG490 is widely used for inhibiting JAK and STAT3 activation (Negoro, Kunisada et al. 2000, Huang, Yang et al. 2010) and it was used here to further study the link between JAK/STAT3 and Fpr2/3.

AG490 (3mg/Kg) (Gross, Hsu et al. 2006, Yan, Jiang et al. 2012), administered 15 minutes prior ischemia, was able to induce Fpr2 mRNA ($p<0.05$). Also AnxA1 mRNA was up-regulated after AG490 administration (Figure 57). It is clear that

when some cardioprotective pathways (like JAK/STAT3) are down regulated, other protective pathways, like Fpr2/3-AnxA1, are up regulated.

AG490 was also able to decrease the cardioprotective activity induced by AnxA1 (40 μg/Kg i.v. prior ischemia) showing that the protein need the activation of the JAK/STAT3 pathway to induce a cardioprotection.

In line with that we detected a injured morphology of the heart with infiltrated cells (Figure 58), and the same infarct size ($p<0.01$ AnxA1 *vs.* AG490+AnxA1) (Figure 59 A) and caspase 3 activity ($p<0.05$ WT AMI or WT AG490+AnxA1 *vs.* WT Sham) (Figure 59 B) in mice treated with vehicle or AG490 and AnxA1. In all cases the effects of AnxA1 was lost when administered together with the JAK/STAT3 inhibitor.

Intravenous injection of AG490 was also able to induce IL-6 ($p<0.001$) mRNA and TNFα mRNA ($p<0.001$) underling the ability of the JAK/STAT3 pathway to induce a negative feedback toward IL-6 production and anti-inflammatory effects (Figure 60).

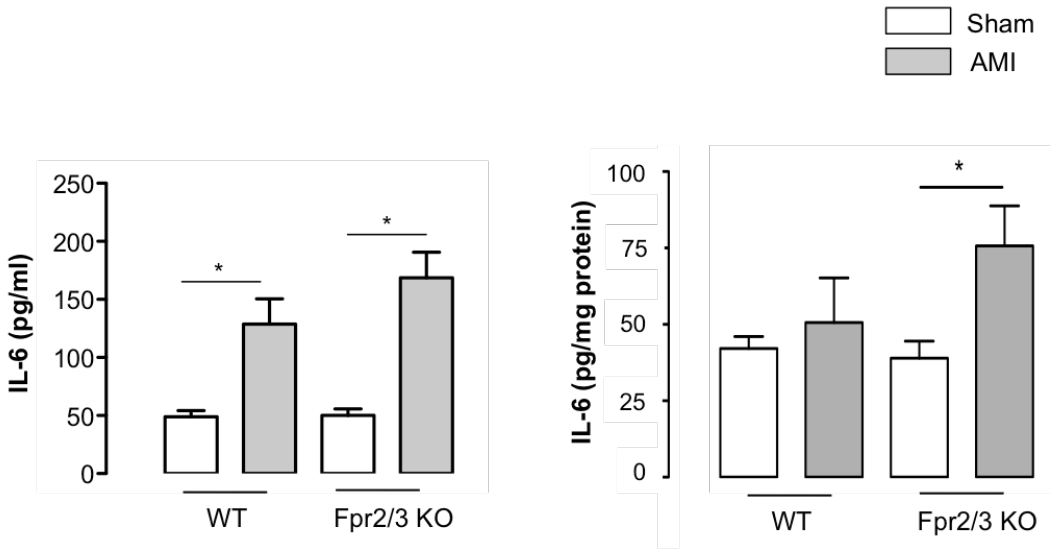


Figure 55: IL-6 levels in plasma and heart of WT and Fpr2/3 KO mice after AMI.
WT and Fpr2/3 KO mice were subjected to LADCA occlusion for 30 minutes and 90 minutes of reperfusion. At the end of the procedure whole blood and heart were collected and analysed by ELISA for IL-6 expression. Acute myocardial infarct was able to increase IL-6 in plasma of both WT and Fpr2/3 KO mice ($p<0.001$). In the heart, IL-6 was induced by AMI only in Fpr2/3 KO mice ($p<0.05$). Data are showed as mean ± SEM of 8 to 15 mice per group.

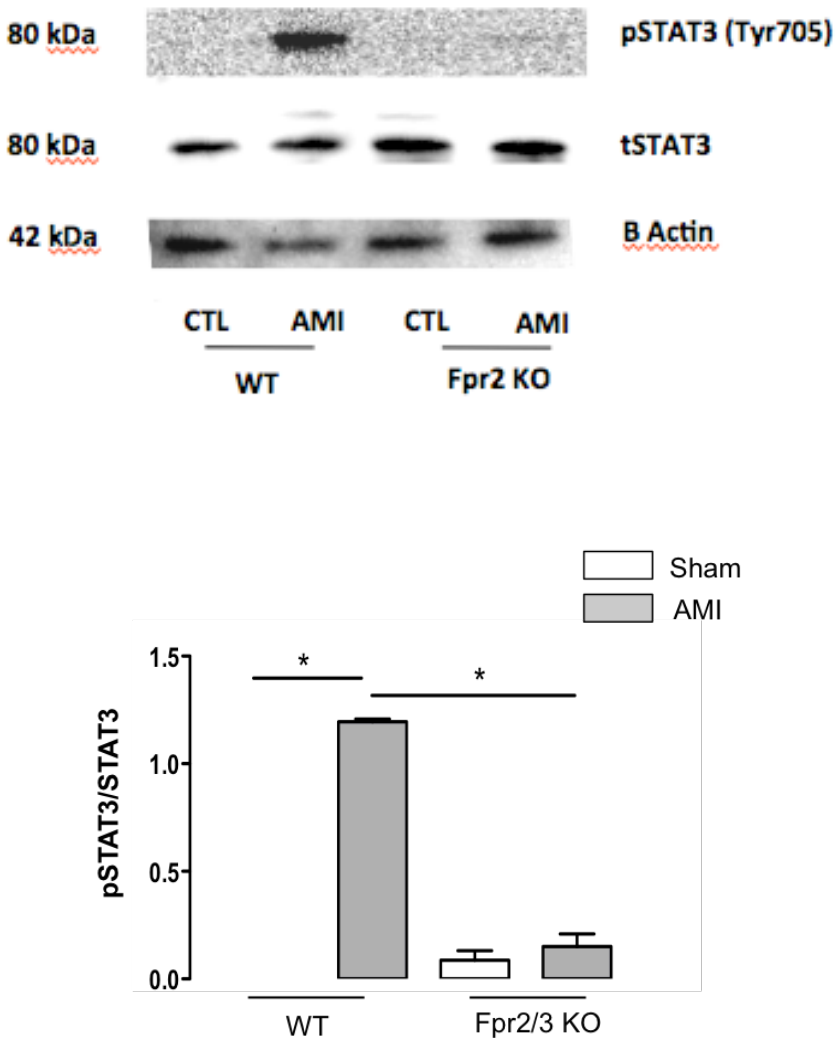


Figure 56: STAT3 phosphorylation by AMI in heart of WT and Fpr2/3 KO mice.
In the heart, pSTAT3 was induced by 30 min of LADCA ligation and 90 min of reperfusion in WT mice ($p<0.001$ *vs.* WT AMI and KO AMI). Protein expression has been monitored by western blot analysis using a pSTAT3 antibody (1:1000) and a STAT3 antibody (1:2000). Representative blots from more than 3 experiments with distinct cell preparations are shown.

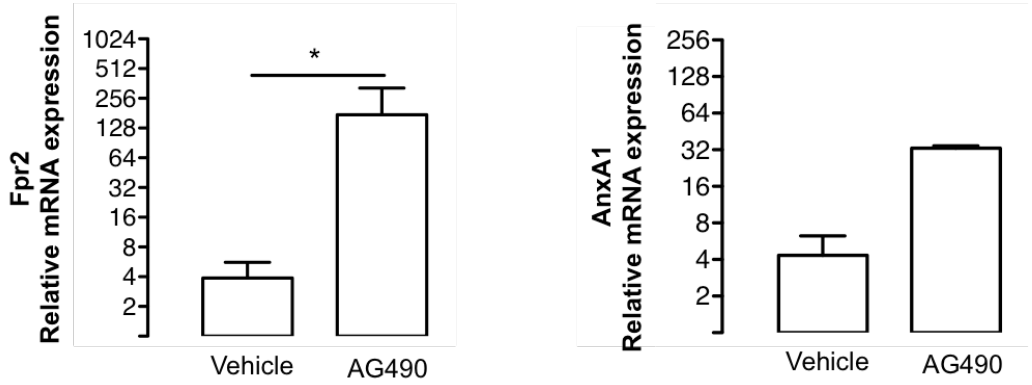


Figure 57: Fpr2 and AnxA1 mRNA expression in heart tissue samples after AMI in the presence of JAK/STAT3 pathway blockade.

WT mice were injected i.v., 15 min prior ischemia, with vehicle (PBS) or AG490 (3mg/Kg) and subjected to 30 min of LADCA occlusion and 90 min of reperfusion. Hearts were then collected and analysed by RT-PCR for Fpr2 and AnxA1 mRNA expression using the relative quantification method for data analysis. AMI induced an increase in Fpr2 and AnxA1 mRNA expression. AG490 increased the induction of Fpr2 mRNA ($p<0.05$ *vs.* Vehicle). The response produced in control mice (no AMI) was considerated as 1 and the data were expressed as an upregulation (>1) or downregulation (<1) of mRNA expression after AMI. GAPDH was used as endogenous control. Data are showed as mean ± SEM of 8 to 15 mice per group.

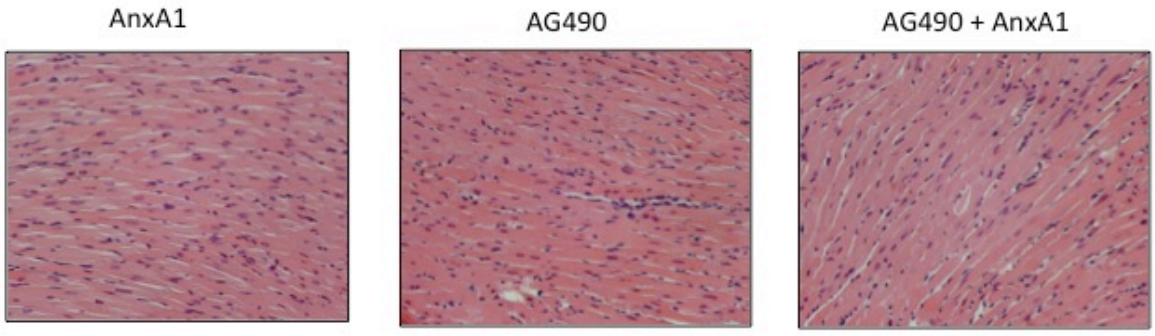


Figure 58: Section of hearts after acute myocardial infarct and a treatment of AnxA1 and/or the JAK/STAT3 inhibitor.

H&E staining of WT mice treated with AnxA1 (40 µg/Kg i.v) prior ischemia, AG490 (3mg/Kg i.v.) 15 minutes prior ischemia or AG490 followed by AnxA1 and following 30 minutes of occlusion of the LADCA and 90 minutes of reperfusion. Hearts were then removed, fixed in PFA, paraffin-embedded and stained with haematoxylin and eosin. Pictures are representative of at least 6 different slices of hearts from 5 to 8 mice per group.

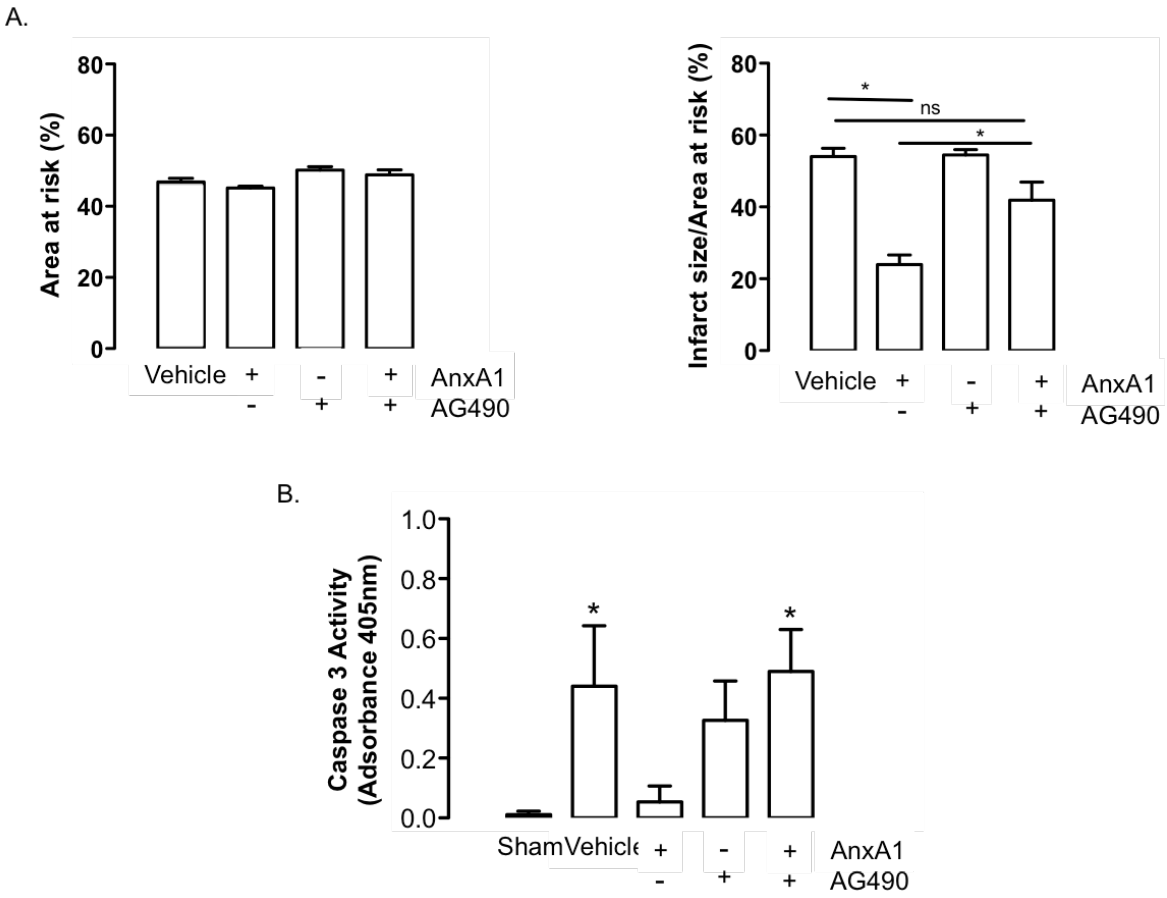


Figure 59: The JAK/STAT3 inhibitor AG490 reduces the cardioprotective activity of AnxA1. WT mice were treated with, vehicle (PBS), AnxA1 (40 µg/kg i.v) prior ischemia, AG490 (3mg/kg i.v.) 15 minutes prior ischemia alone or in combination with AnxA1. The infarct size and the caspase 3 activity were valuated after 30 minutes of ischemia and 90 minutes of reperfusion. (A) AMI caused ~55% necrosis of the left ventricle (Area at risk) in all conditions. AnxA1 decreased the infarct size, shown related to the area at risk, induced by AMI ($p<0.01$). AG490 reversed this effect ($p<0.05$). (B) AMI induced the activity of caspase 3 ($p<0.05$). AnxA1 was no longer active when injected with AG490 ($p<0.05$ WT AG490+AnxA1 *vs.* WT Sham). Data are expressed as mean ± SEM of 6 to 10 mice per group.

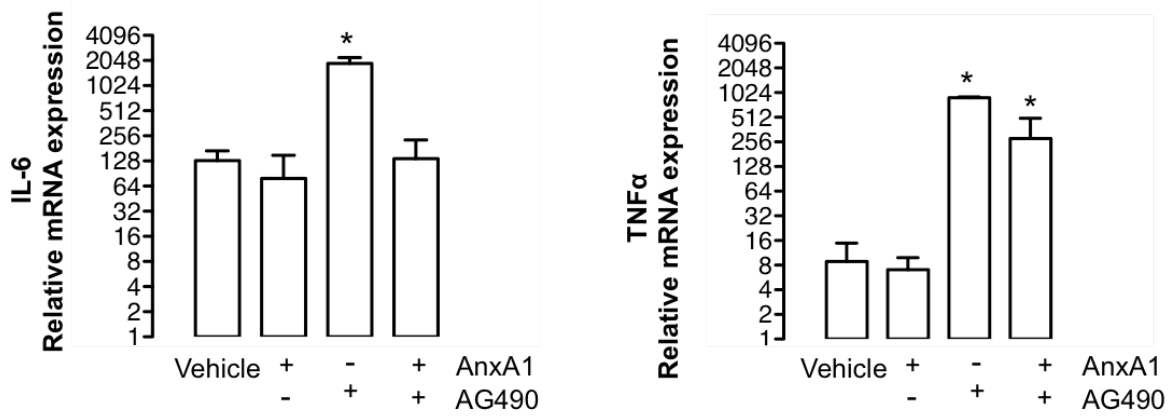


Figure 60: Hearts TNFα and IL-6 production by AMI with or without AnxA1 and AG490 treatment.

WT mice were subjected to myocardial infarct surgery (30+90 min I/R of the heart) and treated with: vehicle (PBS), AnxA1 (40 µg/Kg i.v) prior ischemia, AG490 (3mg/Kg i.v.) 15 minutes prior ischemia or AG490 followed by AnxA1. At the end of reperfusion hearts were collected and analysed by RT-PCR using the relative quantification method for mRNA analyses. AG490 induced IL-6 ($p<0.001$ *vs.* vehicle, AnxA1, and AnxA1+AG490 treatment) and TNFα ($p<0.001$ *vs.* vehicle and AG490 and AnxA1 treated mice) mRNA. TNFα mRNA was also induced with the treatment of AG490 and AnxA1 ($p<0.001$ *vs.* vehicle and AnxA1 treated mice). mRNA data are compared with sham mice (taken as 1) and expressed as upregulation (>1) or downregulation (<1) of mRNA. GAPDH was used as endogenous control. Data are mean ± SEM of 6 to 10 mice per group.

4.5 Role of FPR2/ALX agonists in AMI

4.5.1 Lipoxin A_4

The role of the FPR2/ALX agonist LXA_4 in acute myocardial infarct was studied. WT mice were treated with 100 ng of LXA_4 (Brancaleone, Gobbetti et al. 2013) prior ischemia and biochemical analysis was performed in heart and plasma of mice at the end of AMI.

Lipoxin A_4 was not able to induce a cardioprotection. Indeed the levels of the infarct size (Figure 61), troponin I and caspase 3 (Figure 62) were the same in mice treated with vehicle and LXA_4 and operated with the AMI protocol. In terms of inflammation LXA_4 decreased the levels of TNFα ($p<0.05$) and IL-6 mRNA ($p<0.05$) induced by AMI (Figure 63). LXA_4 did not have any effects on the increase of KC and IL-6 induced by acute myocardial infarct (Figure 63).

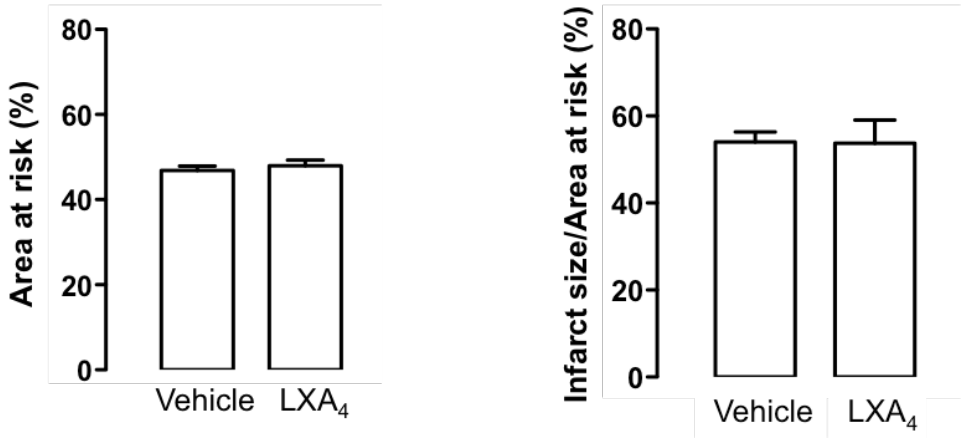


Figure 61: Effect of Lipoxin A_4 on the infarct size caused by acute myocardial infarct.
After 30 min of Ischemia and 90 min of reperfusion WT mice treated with vehicle (PBS) or LXA_4 (100ng prior ischemia) showed the same level of area at risk (50%) and infarct size (55%). Infarct sizes are shown related to the area at risk. Data are expressed as mean ± SEM of 6 mice per group.

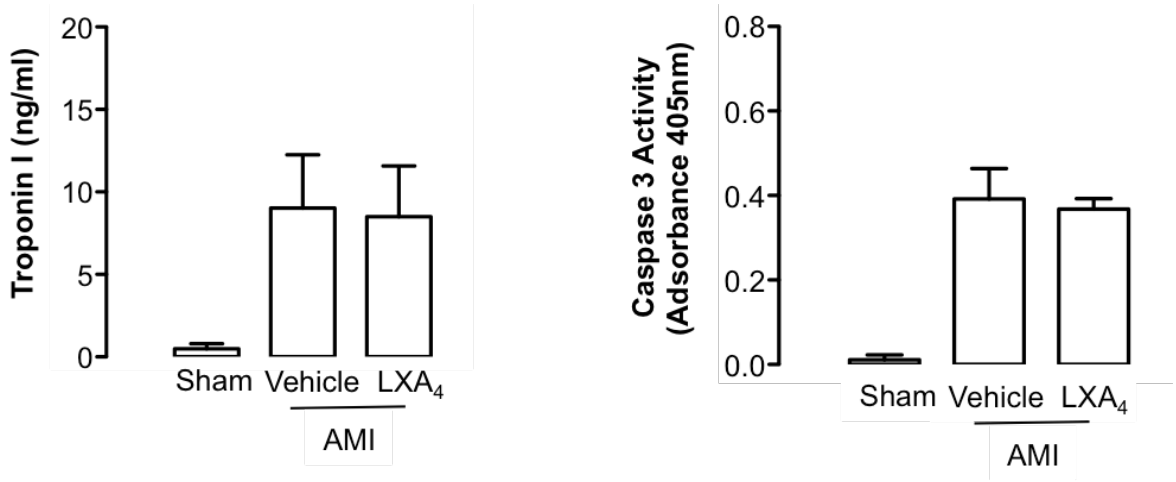


Figure 62: Effects of LXA_4 on cardiac injury biomarkers after AMI.

WT mice were subjected to LADCA occlusion for 30 minutes followed by 90 minutes of reperfusion. At the end of the procedure whole blood and hearts were collected and analysed by ELISA for troponin I levels and caspase 3 activity. LXA_4 (100ng) administrated i.v prior ischemia has not changed the levels of Troponin I ($p<0.05$) and Caspase 3 ($p<0.05$) induced by acute myocardial infarct. Data are showed as mean ± SEM of 6 mice per group.

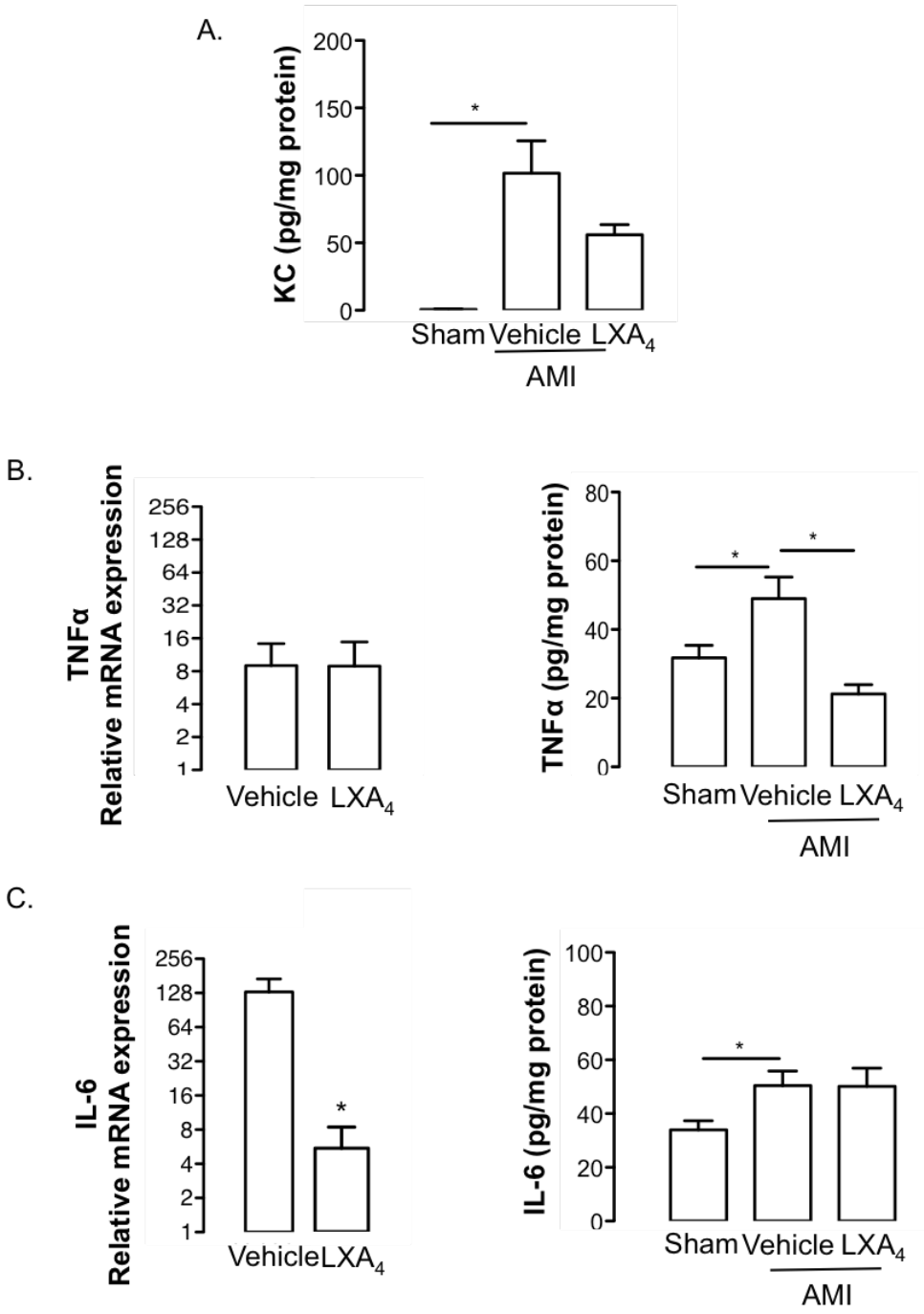


Figure 63: Role of LXA_4 on the inflammatory response induced by AMI in the heart.
WT mice were treated i.v with vehicle (PBS) and LXA_4 (100ng) prior 30 min of ischemia. At the end of 90 min of reperfusion hearts were excised and levels of KC, TNFα and IL-6 were analysed. (A) KC levels were upregulated by AMI with both vehicle ($p<0.05$) and LXA_4 treatments. (B) TNFα was upregulated in terms of mRNA (left panel) and proteins (right panel) ($p<0.05$). LXA_4 reduced the increase of TNFα ($p<0.05$) induced by AMI. (C) I/R of the heart upregulated IL-6 mRNA and proteins expression ($p<0.05$). LXA_4 reduced the increase of IL-6 mRNA ($p<0.05$). Following the relative quantification method, mRNA data are compared with sham mice (taken as 1) and expressed as upregulation (>1) or downregulation (<1) of this value. GAPDH was used as endogenous control. Data are mean ± SEM of 6 mice per group.

4.5.2 Resolvin D1

The role of Resolvin D1, as a novel FPR2/ALX agonist (Krishnamoorthy, Recchiuti et al. 2010), was also studied in AMI. WT mice were treated with RvD1 prior 30 minutes of ischemia and 90 minutes of reperfusion. Heart and plasma were then collected and analysed together with the quantification of the infarct size.

Treatment of mice with RvD1 (100 ng i.v.(Norling, Dalli et al. 2012) prior ischemia was able to decrease the infarct size induced by AMI ($p<0.005$) (Figure 64); however this macroscopic effect on tissue injury was not paralleled by change in the induction of troponin I and caspase 3 activity (Figure 65). In contrast, RvD1 decreased circulating levels of KC ($p<0.001$) and IL-6 mRNA ($p<0.001$) increased after acute myocardial infarct (Figure 66). No effects of TNFα and IL-6 modulation were detected (Figure 66).

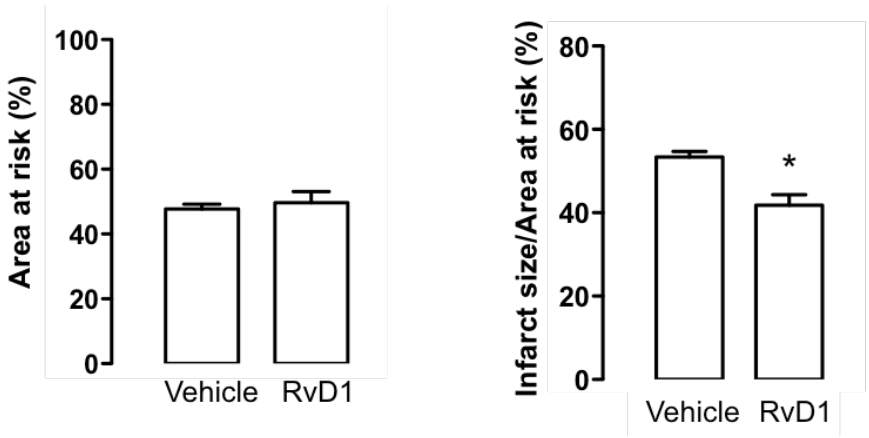


Figure 64: Effect of resolvin D1 in the infarct size induced by acute myocardial infarct. WT mice were subjected to 30 min of ischemia through occlusion of the LADCA and 90 min of reperfusion. All mice were injected intravenously with vehicle (PBS) or RvD1 (100 ng) prior ischemia. At the end of reperfusion the area at risk (AAR) and the infarct size were analysed. LADCA occlusion induced ~55% necrosis of the AAR in all mice. RvD1 treatment significantly decreased the infarct size, showed related to the area at risk, compare with vehicle ($p<0.05$). Data are showed as mean ± SEM of 8 mice per group.

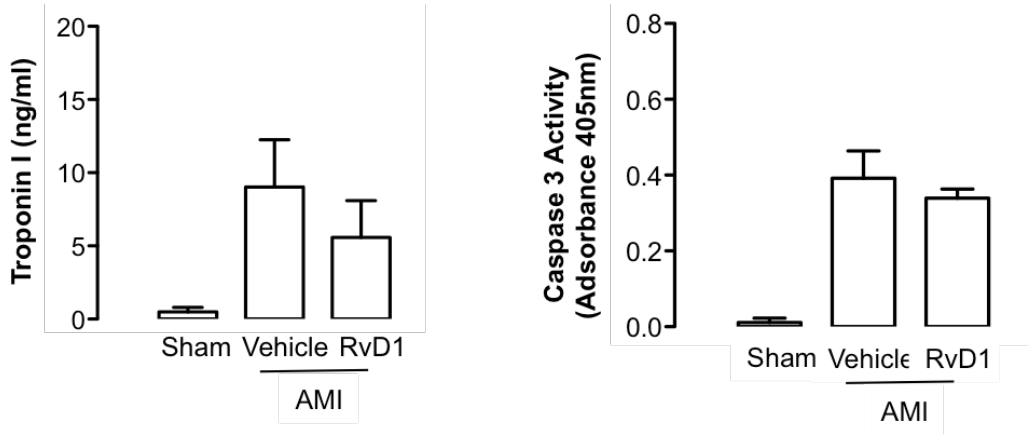


Figure 65: Effects of resolvin d1 on cardiac injury biomarkers after AMI.
WT mice were treated with an i.v injection of vehicle (PBS) or RvD1 (100ng) prior 30 min of ischemia and 90 min of reperfusion. At the end of the AMI surgery whole blood and plasma were collected and analysed by ELISA. WT mice treated with vehicle or RvD1 showed the same levels of Troponin I ($p<0.05$) and Caspase 3 in the heart ($p<0.05$) induced by acute myocardial infarct. Data are showed as mean ± SEM of 8 mice per group.

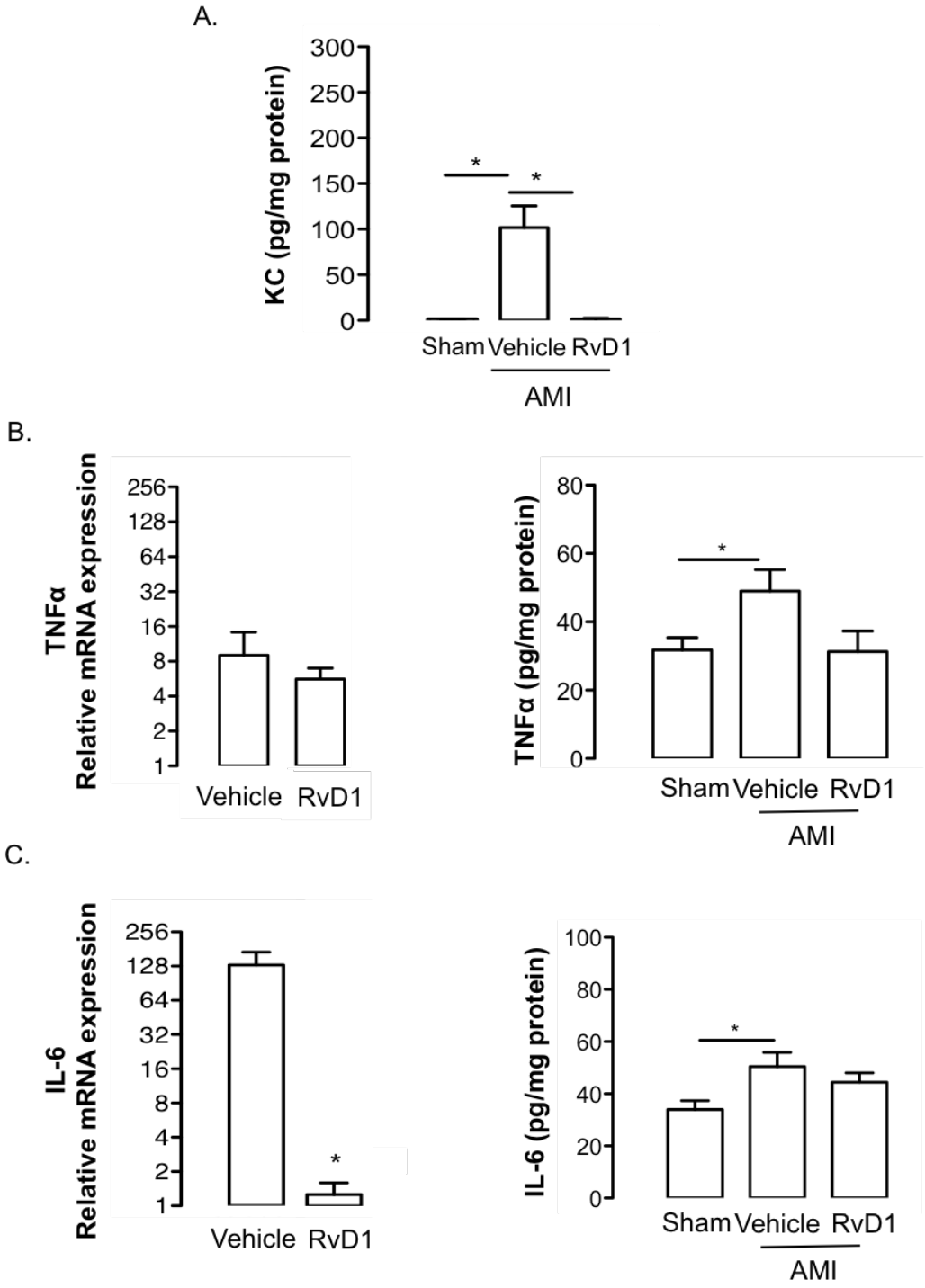


Figure 66: Role of RvD1 on the inflammatory response induced by AMI in the heart. WT mice were treated with vehicle and i.v injection of RvD1 (100ng) prior 30 min of ischemia. At the end of reperfusion hearts were removed and KC, TNFα and IL-6 levels studied. (A) KC was upregulated by AMI ($p<0.05$) and reduced by RvD1 ($p<0.05$). (B,C) TNFα and IL-6 were upregulated both in terms of mRNA (left panel) and proteins (right panel) ($p<0.05$) expression. RvD1 reduced the levels of IL-6 mRNA upregulated by AMI ($p<0.001$). Data are showed as mean ± SEM of 8 mice per group.

4.5.3 Compound 43

To compare the effects of endogenous and synthetic FPR2/ALX agonist of AMI, the AMGEN compound 43 was administered i.v or i.p prior 30 minutes of ischemia. At the end of 90 minutes of reperfusion the infarct size was analysed.

C43 was injected i.v (10µg, 90µg, 150µg) prior ischemia or i.p at 10µg 30 minutes prior ischemia.

All the doses of C43, given i.v., were able to decrease the infarct size induced by AMI ($p<0.05$) in the same pattern (Figure 62): the effect was not dose dependent. C43 injected i.p was not able to change the infarct size induced by AMI (Figure 67). When compared with the activity of AnxA1 on AMI the effect of C43 was significantly lower ($p<0.001$) (Figure 68).

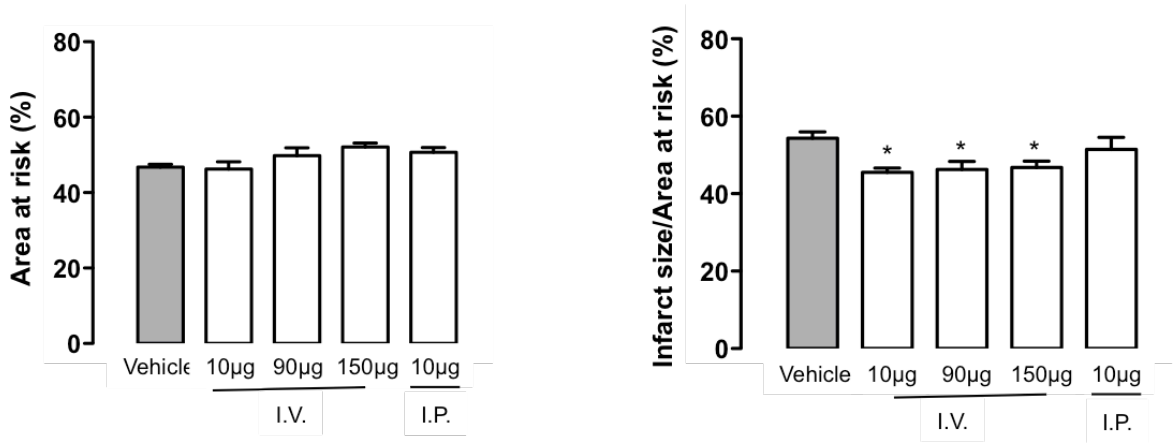


Figure 67: Effect of different doses of compound 43 in the infarct size caused by acute myocardial infarct.

During AMI (30+90 min I/R of the heart through LADCA occlusion) WT mice were treated with different doses of C43. Specifically, C43 was injected i.v (10µg, 90µg, 150µg) prior ischemia or i.p at 10µg 30 minutes prior ischemia as shown. At the end of reperfusion the infarct sizes, referred to the area at risk, were analysed. Mice treated with the i.v injections of C43 showed a decrease in infarct size compared with mice treated with vehicle ($p<0.05$). C43 given i.p was not able to induce a cardioprotective effect. Data are showed as mean ± SEM of 6 mice per group.

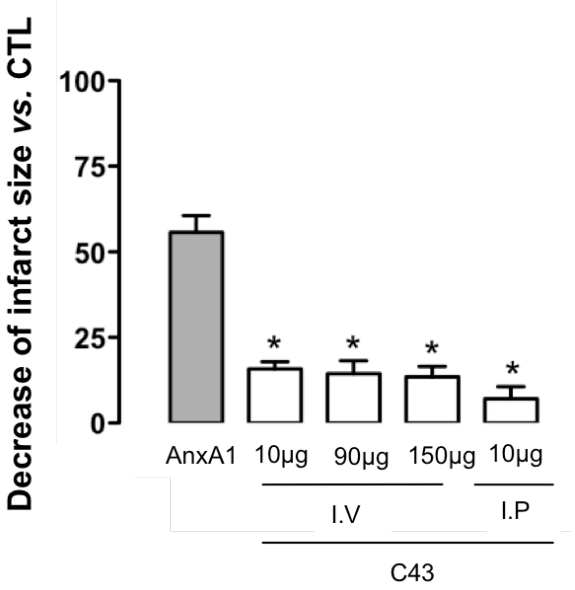


Figure 68: Comparison between the effects of AnxA1 and C43 on AMI.
The decrease of infarct size induced by showed doses of C43 in WT mice was significantly lower ($p<0.001$) in comparison with the one induced by AnxA1 (1 µg) administrated i.v prior ischemia. Data are showed as mean ± SEM of 6 mice per group.

4.5.4 Summary

In summary, as showed in table 4, LXA_4 was not able to induce a cardioprotective effect in WT mice. The lipid only decreased the levels of TNF-α induce by AMI.

RVD_1 decreased the infarct size and the increase in KC levels induced by LADCA occlusion.

All the doses of C43, injected i.v., decreased the infarct size induced by AMI in a not dose dependent way. The other markers of cardiac injury or inflammation were not analysed for this synthetic compound.

	Infarct size	Troponin I	Caspase 3	KC	TNF-α	IL-6
LXA_4					X	
RvD_1	X			X		
C43	X	-	-	-	-	-

Table 4: Summary of LXA4, RvD1 and C43 effects on acute myocardial infarct in WT mice
WT mice were treated with LXA_4 (100ng prior ischemia), RvD_1 (100ng prior ischemia) and C43 (10µg, 90µg, 150µg i.v. prior ischemia or 10µg i.p. 30 minutes prior ischemia). LXA_4 induced a decreased in TNF-α and RvD_1 decreased the infarct size and KC levels. Only i.v. injections of C43, were able to decrease the infarct size induced by AMI.

CHAPTER 5: IDENTIFICATION OF FPR2/ALX DOMAINS REQUIRED FOR AGONISTS BINDING AND SIGNALLING

5.1 Rationale

G protein-coupled receptors (GPCRs) are the largest family of membrane receptors with more than 800 of these proteins encoded in the human genome. (Hendriks-Balk, Peters et al. 2008). GPCR are responsible for the regulation of different physiological processes. One is the control of the inflammatory reaction (Sun and Ye 2012), both in terms of promoting the response (e.g. chemokine-mediated leukocyte recruitment) and limiting its duration and intensity. However, more than 100 different GPCRs are expressed in the cardiovascular system and their cardioprotective activity is emerging (Wieland, Lutz et al. 2007, Waterson, Thompson et al. 2011). Their role is accomplished via ligand binding to GPCRs, activating associated G proteins and intracellular signalling pathways.

Within the context of inflammation, the GPCR FPR2/ALX receptor together with its agonist AnxA1, might play an important role, as we shown in this study, in acute myocardial infarct. AnxA1 mimetic might be developed to target acute myocardial injury.

However FPR2/ALX is an intriguing receptor: it is the first one that mediates actions elicited by both lipids, peptides and proteins, but also it mediate pro-inflammatory responses, such as those elicited by its agonist serum amyloid protein A (SAA). A recent study by Ye's group chiefly demonstrates the ability of FPR2/ALX to modulate cell responsiveness in a ligand-biased fashion (Li, Cai et al. 2011).

For this reason, the adverse effect mediated by the receptor by different FPR2/ALX agonists must be considered. To address this problem, an AnxA1-biased agonist might result in unique receptor activation that will signal to desirable pathways while sparing those pathways mediating undesirable effects.

Several mechanistic hypotheses can be put forward to explain the versatility of the FPR2/ALX receptor, including homo- and/or hetero-dimerization as well as the recruitment of ligand specific signalling pathways. Another plausible option is that ligands might activate specific receptor domains thus promoting at least in part not overlapping downstream responses. As an example, the small lipid lipoxin A_4 has been shown to activate FPR2/ALX by interacting with the III extra-cellular loop and associated trans-membrane region (Chiang, Serhan et al. 2006).

For this reason we decided to study the domains required for AnxA1 and SAA-dependent receptor activation. For completion and to assess the potential impact these finding might have on drug discovery, Compound 43, a synthetic pro-resolving agonist, has also been studied. To do so, we took advantage of recently characterized stable clones expressing chimeric receptors with different regions of FPR2/ALX and the pro inflammatory receptor FPR1 (Le, Ye et al. 2005).

5.2 Native and chimeric human FPR1 and FPR2/ALX receptors

Human FPR1 and FPR2/ALX receptors display ~69% homology (Ye, Boulay et al. 2009) as illustrated in Figure 69 where, in the human FPR2/ALX sequence, the amino acids different from human FPR1 are highlighted in red.

To evaluate the contribution of single domains in FPR2/ALX activation by selective agonists we used eight different chimeric receptors with swapped domains between FPR1 and FPR2/ALX, generated as described in (Le, Ye et al. 2005), and detailed in Figure 70. The degree of receptor expression of each chimaera was assessed using specific monoclonal antibodies (mAb) against FPR1 and FPR2/ALX. As shown in figure 71, only Chimaera C, D and G were recognized by the anti-FPR2/ALX mAb, whilst all others clones were detected with the anti-FPR1 mAb.

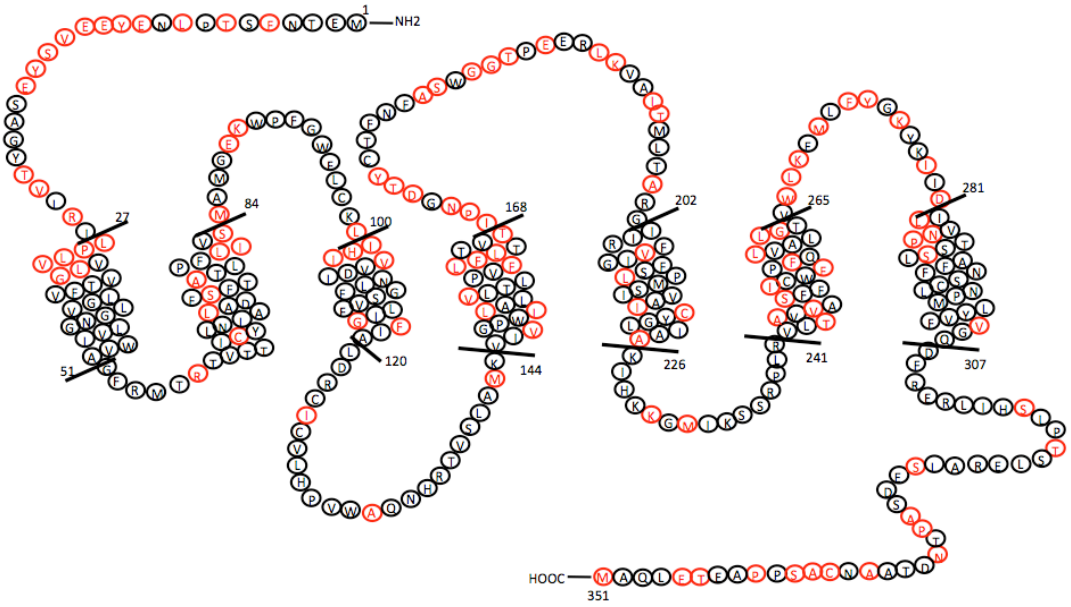


Figure 69: Human FPR2/ALX amino acids sequence.
Representation of the sequence of the human FPR2/ALX receptor with amino acids different from the human FPR1 receptor highlighted in red.

	FPR1 sequence	FPR2 sequence
FPR1	1 - 350	-
FPR2	-	1 - 351
Clone A	1 - 294	295 - 351
Clone B	1 - 240	241 - 351
Clone C	40 - 351	1 - 39
Clone D	1 – 39 87 - 351	40 - 86
Clone E	1 – 86 106 - 351	87 - 105
Clone F	1 – 105 146 - 351	106 - 145
Clone G	40 - 145	1 – 39 146 - 351
Clone H	40 – 145 241-351	1 – 39 146 - 240

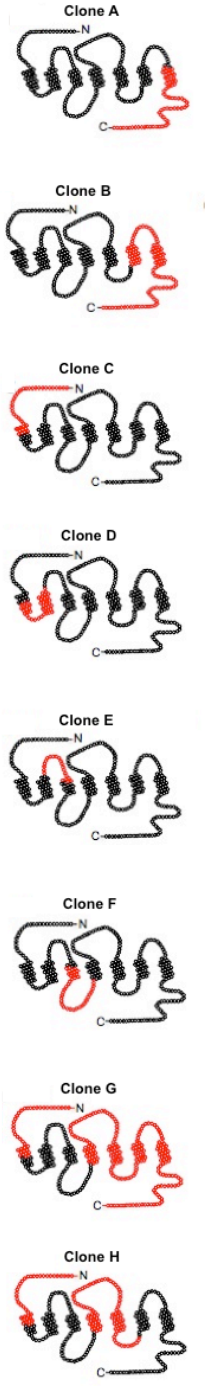
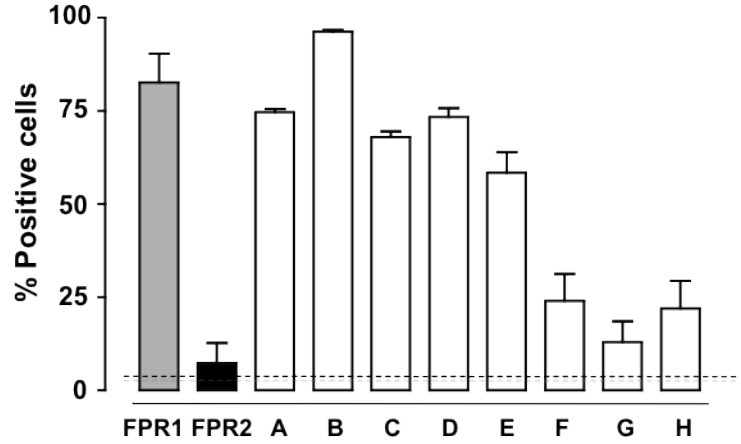


Figure 70: Schematic representation of chimeric human FPR1 and FPR2/ALX clones. FPR1-FPR2/ALX clones used in this study. Each clone is named alphabetically (A,B,C,D,E,F,G,H) and has distinct domains of the FPR1 receptor replaced with the indicated FPR2/ALX amino acid sequences (in red-right panel). Amino acids sequence (left panel) and representative receptor (right panel) are showed

A.



B.

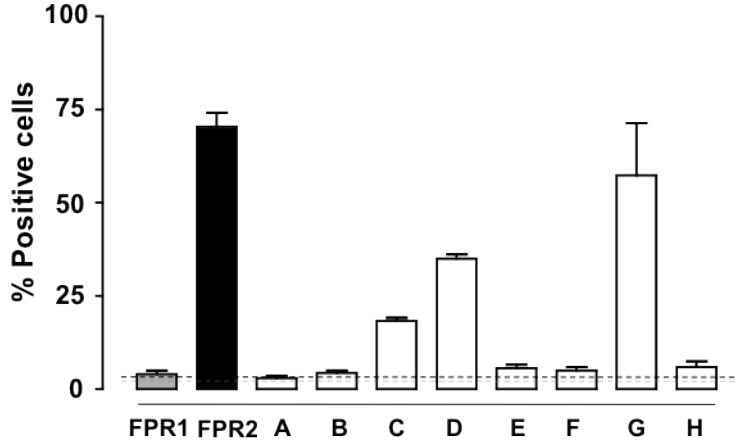


Figure 71: Receptor expression of native and chimeric human FPR1 and FPR/ALX transfected in HEK293 cells.

FPR1 and FPR2/ALX cell surface expression on HEK293 cells transfected with native receptors and the clones was analysed by flow cytometry using the anti-FPR1 (A) or anti-FPR2 (B) monoclonal antibody (1:100 dilution in both cases). Dashed lines indicate antibody interaction with HEK293 cells transfected with empty CMV plasmid. Flow cytometry analysis was performed by analyzing ≥10,000 events using a FACSCalibur flow cytometer equipped with CellQuest software followed by analysis using FlowJo. Data are expressed as median fluorescence intensity units. Data are expressed as means ± S.E.M. of experiments conducted at least 3 times.

5.3 Clones-specific calcium mobilization induced by AnxA1, SAA and C43

AnxA1 and its bioactive peptides are able to mobilize intracellular Ca^{2+} in primary PMN (Walther, Riehemann et al. 2000, Solito, Kamal et al. 2003) and cells transfected with FPR2/ALX and/or FPR1 (Ernst, Lange et al. 2004).

In native FPR2/ALX transfected cells, AnxA1, SAA and C43 induced a concentration-dependent responses (Figure 72).

To compare the ligand-binding domains activated by the different agonists, Ca^{2+} mobilization in FPR1/FPR2/ALX chimaeric HEK-293 cells was studied with concentrations selected from figure 72.

AnxA1 (10 nM) induced Ca^{2+} flux in native FPR2/ALX cells with similar responses in clones C, G and H (all ranging from 40 to 60% of the maximal response). Little or no effect on FPR1 expressing cells and the other clones (Figure 73) were detected.

SAA (100 nM) afforded ~50% of the ionomycin response both with native FPR2/ALX cells and clones E, F, G, and H, whilst no responses were detected with the other clones, including clone B that bears the III extracellular domain of FPR2/ALX (Figure 73).

The synthetic molecule C43 (1 μM) induced a calcium flux in FPR2/ALX transfected cells and only active in clones E and F (Figure 73).

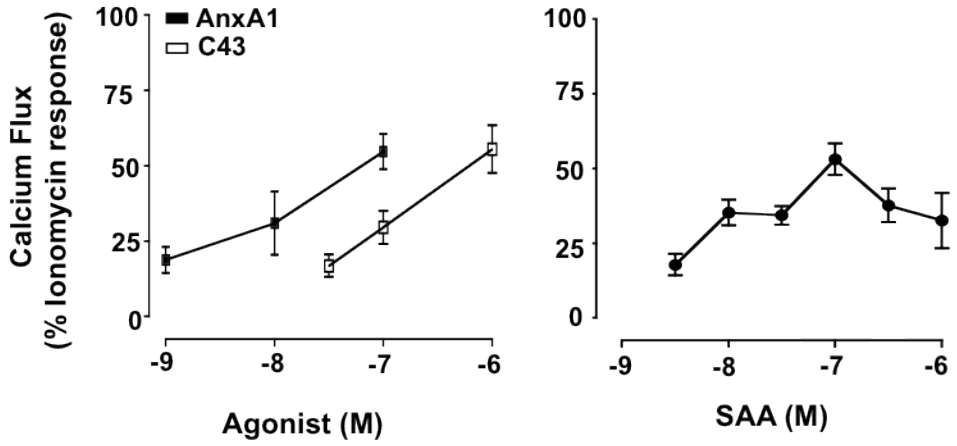


Figure 72: Dose response induced by AnxA1, Compound 43 and SAA in HEK293 FPR2 cells. Ca2+ flux responses evoked in FPR2/ALX transfected HEK293 cells upon treatment with showed concentration of AnxA1 or C43 (left panel) and with SAA (right panel). HEK293 cells were loaded with FURA-2 (2 μM), washed and added to 96-well plates prior to stimulation. Fluorescence (Ca^{2+} fluxes) was analysed with a NOVOstar™ microplate reader (BMG Labtech). Data are reported as % of Ionomycin (1 μM) response and expressed as means ± S.E.M. of experiments conducted at least 3 times.

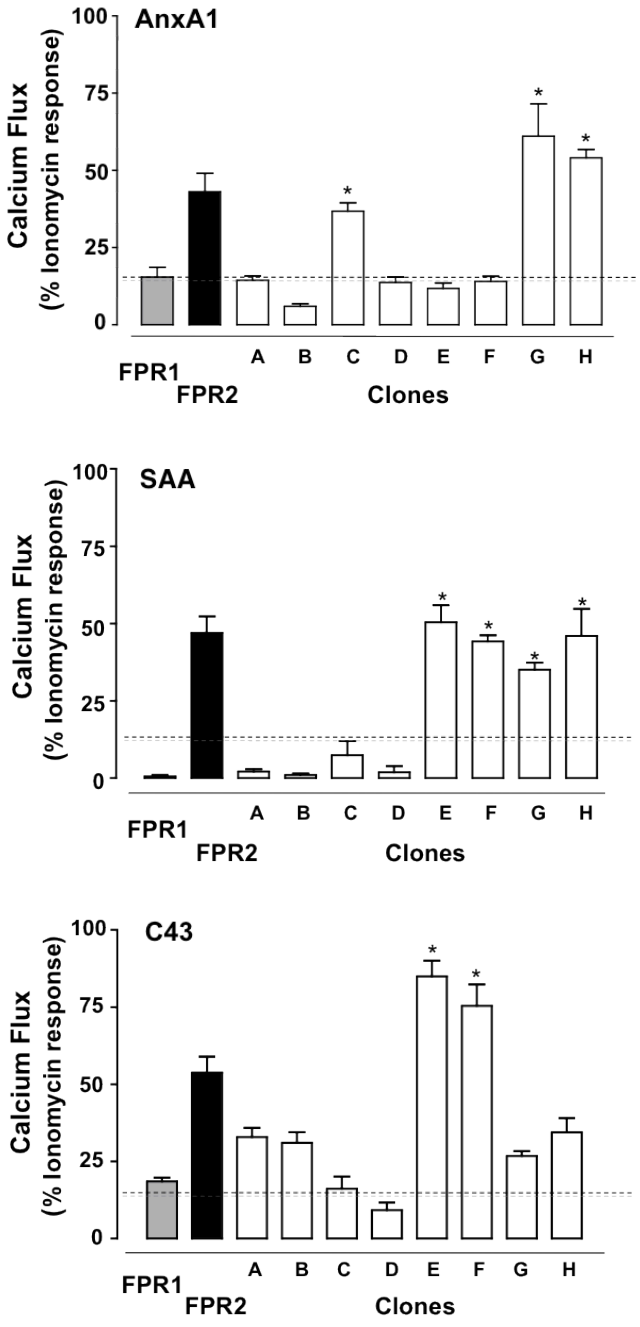


Figure 73: Calcium mobilization induced by AnxA1, SAA and C43 in HEK293 cells transfected with the chimaeric receptors.

FURA-2 loaded HEK293 cells expressing native and chimaeric clones were treated with AnxA1 (10nM), SAA (0.1 μM) or C43 (1 μM) and calcium flux was detected using a fluorescence microplate reader. (A) AnxA1 induced Ca^{2+} flux in native FPR2/ALX cells and clones C, G and H. (B) SAA induced a response in FPR2/ALX cells and clones E, F, G, and H. (C) C43 induced a Ca^{2+} flux in FPR2/ALX transfected cells and in clones E and F. Dashed lines indicate the degree of response produced with control CMV-empty plasmid transfected HEK293 cells in presence of the respective ligand. Data, reported as % of Ionomycin (1 μM) response, are expressed as means ± S.E.M. of experiments conducted at least 3 times. Asterisks indicate similarity in responses between each clone (as indicated) and native FPR2/ALX transfected cells.

5.4 Clones-specific ERK1 and ERK2 phosphorylation induced by AnxA1, SAA and C43

Another readout for the activation of the FPR2/ALX receptor is the phosphorylation of ERK (Lala, Gwinn et al. 1999) and can be used to understand if the clone-specificity that emerged with the calcium flux is linked with other receptor induced signalling pathways. For these reasons native and chimaeric receptors were treated with AnxA1, SAA and C43 with the concentration used for the calcium flux and ERK1/2 phosphorylation analysed by western blotting.

Incubation with AnxA1 (10 nM) for 5 or 10 minutes provoked clear ERK phosphorylation both in FPR2/ALX cells and in clones C, G and H (Figure 74 A). As a negative control, since it was un-responsive to AnxA1 for the Ca^{2+} flux, clone E was also tested and the same inactivity held true for phospho-ERK.

Also SAA, like for the calcium flux response, induced ERK phosphorylation in FPR2/ALX cells as well in clones C, G and H (Figure 74 B). The positive association between Ca^{2+} flux and phospho-ERK was also observed for C43 (1 μM), since it activated both the native receptor and clones E and F (Figure 74 C).
As sometimes evident with transfected cells, the over-expressed receptor was in some instances already partially activated.

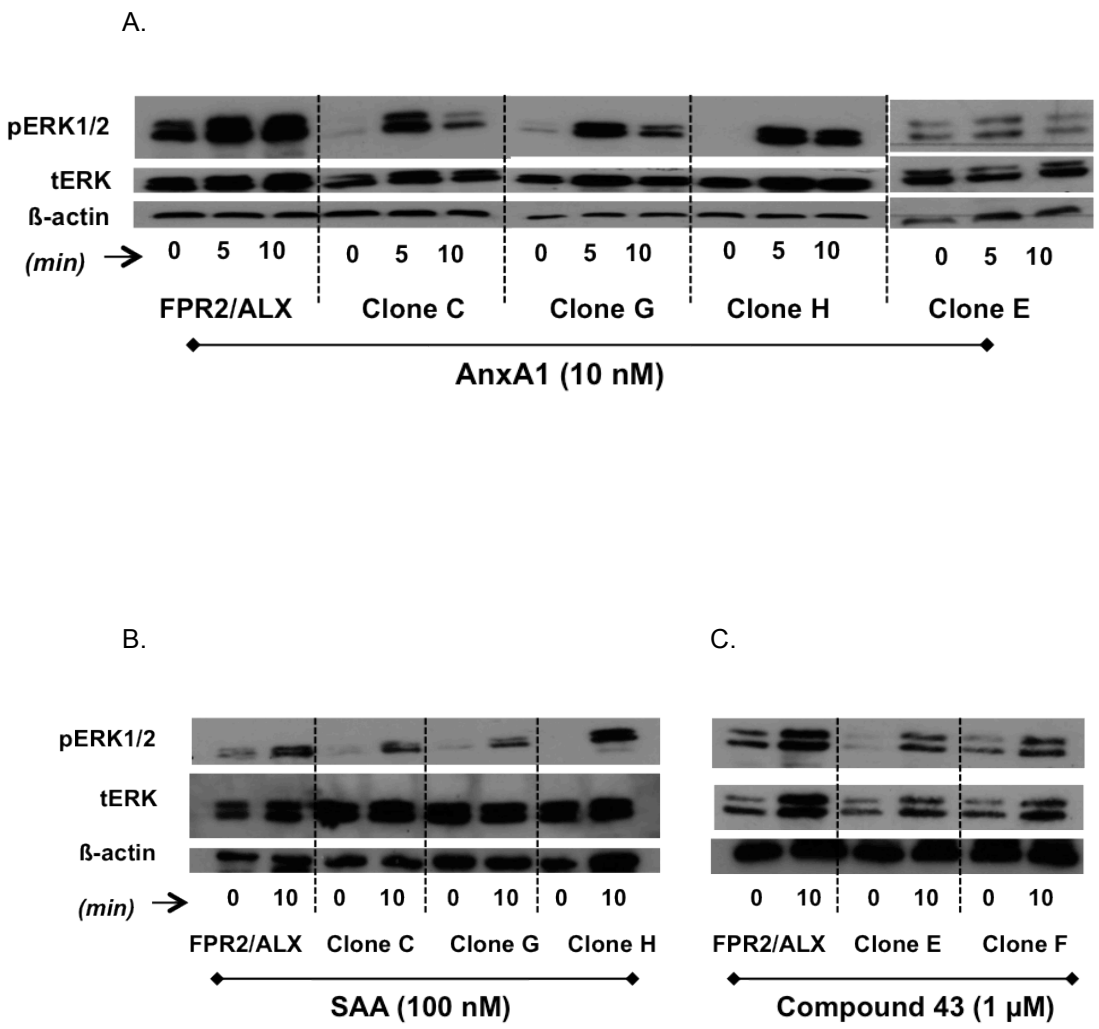


Figure 74: Clone-specific ERK1/2 phosphorylation induced by AnxA1, SAA and C43. HEK293 cells expressing native FPR1 and FPR2/ALX receptor and chimaeric clones were treated with (A) AnxA1 (10nM), (B) SAA (0.1 µM) or (C) C43 (1 µM) for 5 or 10 min, as indicated, and the phosphorylation of ERK1/2 monitored by Western blot analysis. (A) AnxA1 induced pERK 1/2 in FPR2/ALX cells and in clones C, G and H. Clone E was used as negative control. (B) SAA activated both the native receptor and clones C, G and H (C) C43 evoked pERK 1/2 in FPR2/ALX cells as well in clones E and F. Representative blots from ≥3 experiments with distinct cell preparations are shown.

5.5 Clone-specific genomic response evoked by AnxA1

Confirmed the FPR2/ALX domains-specificity for AnxA1, SAA and C43 using the calcium flux and ERK1/2 phosphorylation we then analysed the genomic response induced by receptor activation. To do so, we focused our attention on AnxA1 since its cardioprotective activity and our interest in the potential of this protein in AnxA1-mimetic synthesis for drug discoveries.

For this reason we tested whether the rapid responses elicited by the FPR2/ALX domains expressed in clones C, G and H upon application of AnxA1 could also lead to genomic responses. Specific profiles of gene expression post-AnxA1/FPR2/ALX interaction has been recently shown with a particular validation for the upregulated *JAG1* (Jagged1) and *SGPP2* (sphingosine-1-phosphate phosphatase type 2), and downregulated *JAM3* (junctional adhesion molecule-3) (Renshaw, Montero-Melendez et al. 2010).

Using FPR2/ALX-HEK 293 cells we confirmed this profile, though JAG1 induction was relatively modest in our set of experiments (Figure 75). Using the chimaeric receptors, clone H afforded SGPP2 induction similar to the native receptor (Figure 75), whereas clone C was the most faithful for *JAG1* induction (Figure 75). Inhibition of JAM3 – strongly evident for the native FPR2/ALX receptor- was replicated in all three clones C, G and H (Figure 75).

Trying to find a link between calcium flux, ERK1/2 phosphorylation and the genomic responses pharmacological inhibitors like, the MEK1 inhibitor PD98059 and the calcium inhibitor BAPTA-AM, were used.

As showed in Figure 76, SGPP2 induction in clone H didn't require calcium mobilization or ERK phosphorylation, pathways that - in contrast - were needed for JAM3 inhibition in clones C and H.

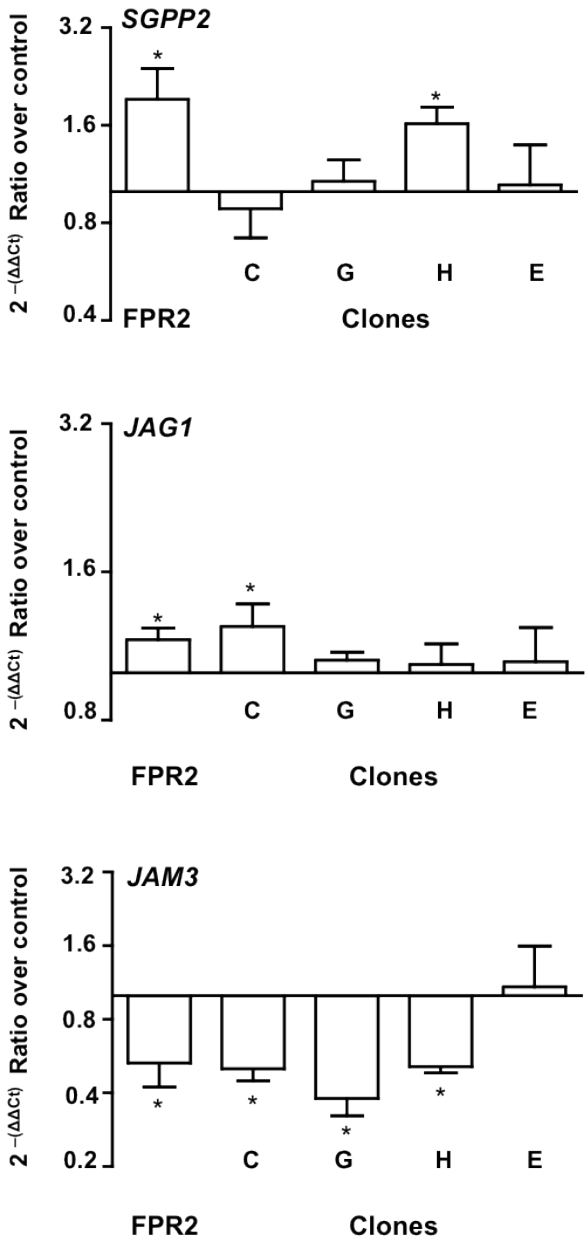


Figure 75: Clone-specific genomic responses evoked by AnxA1 in native and chimaeric receptors.

Real time PCR data for *SGPP2*, *JAG1* and *JAM3* gene expression in HEK293 cells transfected with native FPR2/ALX or the indicated chimaeric clones after incubation with AnxA1 (0.5 μM) for 4h. SGPP2 was induced in FPR2/ALX cells and clone H, JAG1 in FPR2/ALX cells and clone C. JAM3 inhibition was evident in FPR2/ALX cells and clones C, G and H. Clone E was used as negative control. Following the relative quantification method, the response produced with control CMV-empty plasmid transfected HEK293 cells is taken as 1 and data expressed as upregulation (>1) or downregulation (<1) of this value. GAPDH was used as endogenous control. Data are mean ± SEM of experiments conducted at least 3 times. (*P<0.05 *vs.* control CMV-HEK cells).

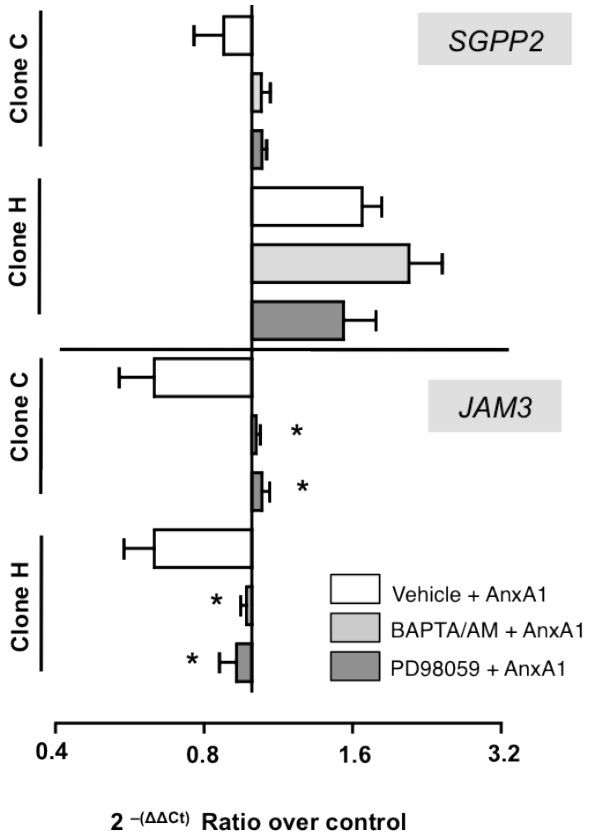


Figure 76: *JAM3* down regulation, but not *SGPP2* upregulation, requires AnxA1-induced calcium flux and ERK phosphorylation.

HEK293 cells expressing native and chimaeric clones C and H were incubated for 30min with either vehicle, the calcium channel inhibitor BAPTA (30μM) or the MEK1 inhibitor PD98059 (10 μM) to prevent phospho-ERK formation prior to addition of AnxA1 (0.5 μM) for further 4 h. SGPP2 induction in clone H didn't require calcium mobilization or ERK phosphorylation. JAM3 inhibition, in clones C and H, was blocked when the inhibitors were used. Gene product expression of *SGPP2* and *JAM3* was then evaluated by real time PCR. The response produced with control CMV-empty plasmid transfected HEK293 cells is taken as 1. Data are mean ± SEM of experiments conducted at least 3 times. (*P<0.05 *vs.* vehicle-treated cells)

5.6 N-terminal domain of FPR2/ALX is dispensable for homologous and heterologous desensitization after agonist binding

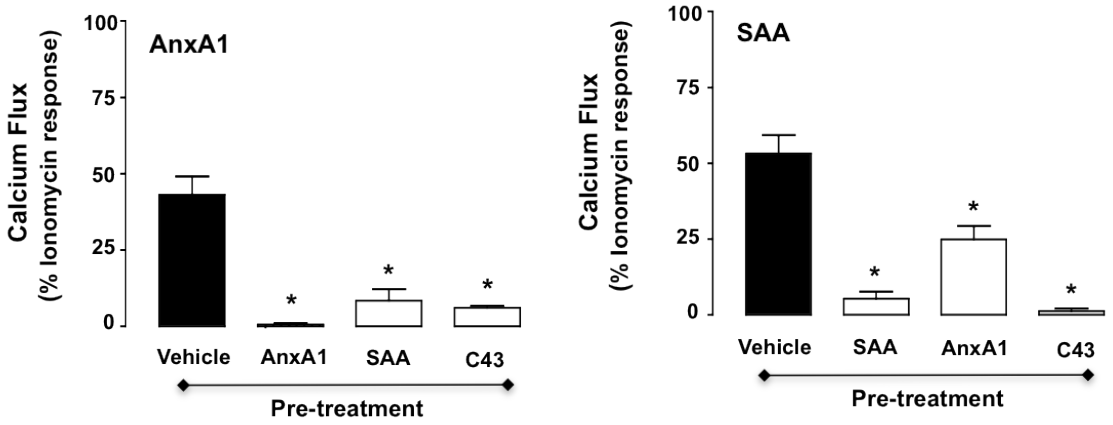
Repeated administration of a drug might evoke a reduction of the response, phenomenon called tolerance. In terms of receptor agonist this might be link with the desensitization in response to multiple activation by one or more agonists.

Since AnxA1 and SAA can induce heterologous desensitization (Dufton, Hannon et al. 2010, Li, Cai et al. 2011) we queried if we could identify the receptor domain required for this biological response.

As showed in Figure 77 A we confirmed the homo- and hetero-desensitization against AnxA1-induced Ca^{2+} flux in FPR2/ALX cells, as exerted by pre-incubation with AnxA1 or SAA, respectively. The same effect was quantified with clones G and H, but not with clone C, indicating that FPR2/ALX N-terminal domain was insufficient to afford desensitization of the AnxA1 response (Figure 78).

To give translation potential to these findings produced with artificial receptors and transfected cells, we studied the cross-desensitization between AnxA1 and SAA in primary neutrophils. SAA (100 nM) desensitized AnxA1-induced Ca^{2+} flux (Figure 77 B) and, conversely, AnxA1 attenuated the SAA response to a similar degree as the homo- desensitization (Figure 77 B). Of interest, when tested at 1 μM C43 also provided modest desensitization on the responses in primary neutrophils (Figure 77 B), whereas it was much more efficient in FPR2/ALX transfected HEK cells (Figure 77 A).

A.



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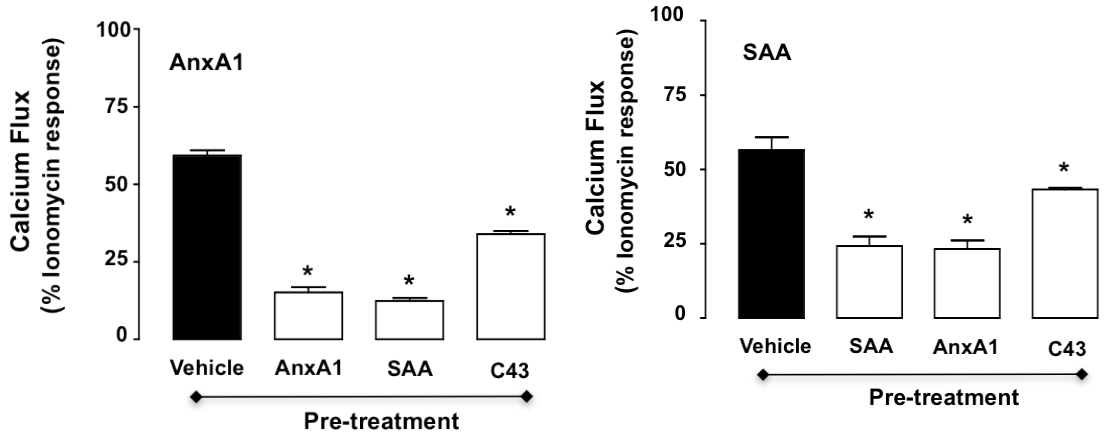

Figure 77: **FPR2/ALX receptor desensitization upon agonist stimulation in HEK293 transfected cells and neutrophils.**

(A) HEK293 cells expressing native FPR2/ALX or (B) freshly isolated human neutrophils were treated with vehicle, AnxA1 (10nM), SAA (0.1 μM) or compound 43 (C43; 1 μM) for 5 min followed by a second treatment with AnxA1 (10 nM) (left panel) or SAA (100 nM) (right panel). All cells were loaded with FURA-2 and the fluorescence was analysed. Homo- and hetero-desensitization was recorded for all the treatment and both cells types. Data are reported as % of Ionomycin (1 μM) response. Data are mean ± SEM of experiments conducted at least 3 times. (*P<0.05 *vs.* native vehicle pretreament).

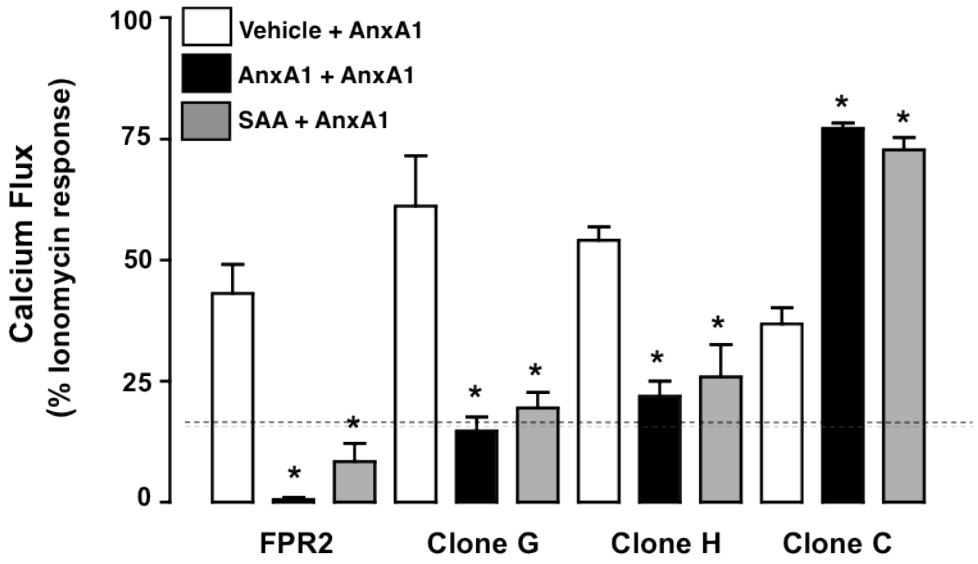

Figure 78: N-terminal domain of FPR2/ALX is dispensable for homologous and heterologous desensitization.

FURA-2 loaded HEK293 cells expressing the FPR2/ALX receptor and clones C, G and H were treated with vehicle, AnxA1 (10nM) and SAA (0.1 μM) for 5 min followed by a second treatment with AnxA1 (10 nM). AnxA1/AnxA1 and SAA/AnxA1 Homo- and hetero-desensitization was confirmed in FPR2/ALX cells, clones G and H but not with clone C. Data are reported as % of Ionomycin (1 μM) response. Data are mean ± SEM of experiments conducted at least 3 times. (*P<0.05 *vs.* Vehicle pretreatment).

5.7 Conclusion

In conclusion this side project allowed me to learn techniques of cell culture and cellular responses in vitro, representing an important complement to the in vivo protocol I had learned plus the biochemical and molecular analyses I conducted in heart tissue samples and in the secondary organs. Furthermore, from a scientific advancement, I have identified AnxA1 distinct domains of FPR2/ALX in relation with other FPR2/ALX agonists like the pro inflammatory SAA and the synthetic anti-inflammatory compound 43.

To summarise, as shown in the figure 79, AnxA1 requires the N-terminus and/or the II extracellular loop to induce FPR2/ALX receptor activation. SAA interacts with I and II extracellular loops for FPR2/ALX, whilst the small synthetic compound 43 penetrates into the I extracellular loop and transmembrane regions for full agonism. The reported binding site for Lipoxin A4 (LXA4) is also indicated, as reported in (Chiang, Fierro et al. 2000).

In terms of activated signalling, AnxA1 thought the FPR2/ALX N-terminal domain induces calcium mobilization and ERK phosphorylation leading to *JAG1* upregulation and *JAM3* downregulation. The II extracellular loop is more complex in its downstream AnxA1-evoked events. It participates in AnxA1 mediated JAM3 downregulation through calcium flux and ERK phosphorylation and it may involve unknown pathways for *SGPP2* induction.

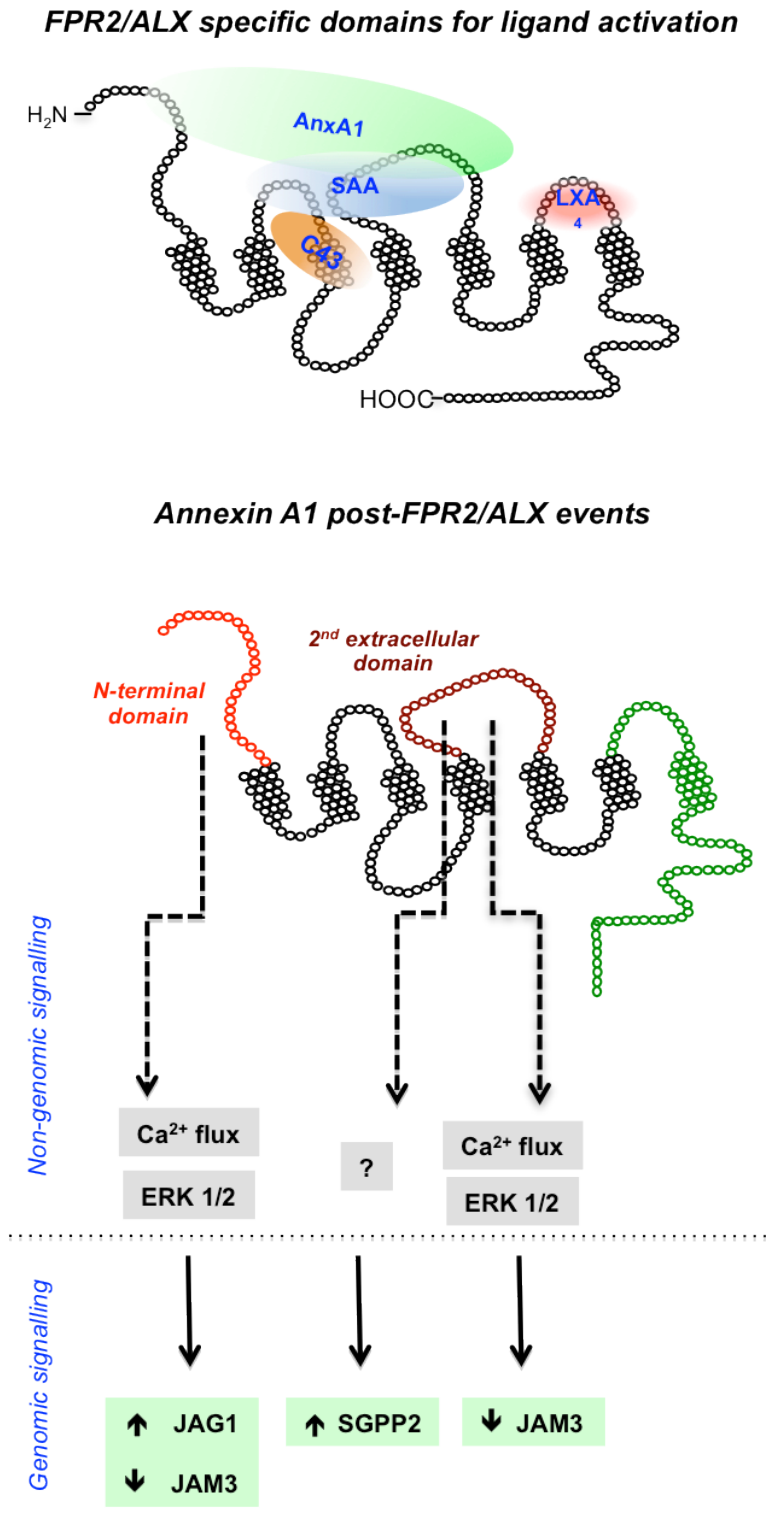

Figure 79: Schematic summary for agonist binding FPR2/ALX and of AnxA1 evoked post-FPR2/ALX signalling.

CHAPTER 6: DISCUSSION

One of the major therapeutic goals of modern cardiology is to design strategies aimed at minimizing myocardial necrosis and optimizing cardiac repair after acute myocardial infarct. However, a deep understanding on the biology of AMI is necessary before a specific intervention is pursued on a therapeutic basis.

Along this way, a growing body of evidence suggests that inflammation play an important role in acute myocardial infarct. It is now clear that the rapid inflammatory response that occurs in the vasculature during AMI is a prerequisite for healing but it can paradoxically also extending tissue injury; hence it needs to be modulated.
An example is the pivotal role of neutrophils: ischaemia–reperfusion injury prompt rapid neutrophil extravasation from the circulation to the infarcted myocardium mediating tissue damage. As we have recently shown (Cash, Bena et al. 2013) reducing neutrophil recruitment can be a successful strategy to decrease heart damage. But this is only a little part of the complex inflammatory response during acute myocardial infarct. Patients with unstable angina and myocardial infarct have also activated circulating neutrophils, lymphocytes, monocytes, increased concentrations of proinflammatory cytokines, such as interleukin IL-1 and IL-6 and coronary atherosclerotic plaques characterized by macrophages at the site of plaque rupture (Biasucci, Liuzzo et al. 2000). Also infiltrating mononuclear cells and mast cells appear to orchestrate the cardiac repair process and the healing phase. They secrete a variety of growth factors and cytokines, inducing fibroblast proliferation (Richard, Murry et al. 1995).

Elucidating the interactions and regulatory mechanisms responsible for myocardial inflammation may help to design effective inflammation-related interventions for the

treatment of myocardial infarction. For this reason appreciation of the mediators operative in the area of resolution of inflammation for AMI is emerging and these include the protein Annexin A1 (AnxA1) and its receptor, the GPCR termed FPR2/ALX.

In line with that, we have recently shown that AnxA1 mediates the inhibition properties of estrogen on PMN activation (Nadkarni, Cooper et al, 2011), female sex hormone linked with a reduced incidence of cardiovascular disease (Bakir, Mori et al. 2000). Furthermore, AnxA1 (D'Amico, Di Filippo et al. 2000) and Annexin-derived peptides such as Ac2-12 and Ac2-26 (La, D'Amico et al. 2001, Qin, Buxton et al. 2012, Qin, Buxton et al. 2013) exert protective effects in rodent models of AMI. *BOC-2*, a pan-FPRs antagonist (able to antagonize FPR1 and FPR2/ALX in human cells, and Fpr1, Fpr2 and Fpr3 in the mouse) reversed the cardioprotective actions of AnxA1 and its N-terminal peptides (La, D'Amico et al. 2001), demonstrating the involvement of FPRs in the cardioprotective properties of these molecules. Ac2-26 improved the recovery of left ventricle function through FPR1 (Qin, Buxton et al. 2013) but retained its fully cardioprotective properties in Fpr1 KO mice (Gavins, Kamal et al. 2005). Thus, FPR2/ALX may be the receptor of the FPRs family with a *protective nature* in AMI.

For these reasons, here, we have investigated the role of the FPR2/ALX receptor in acute myocardial infarct. We explored the damage in the heart and in secondary organs such as the lung and the kidney taking advantage of a unique Fpr2/3 KO mouse colony with a green fluorescence protein (GFP) gene ‘knocked-in’.

Transgenic mice lacking for the Fpr2 and Fpr3 genes compose our mouse colony. This is because the human ALX/FPR2 receptor exhibits homology with 2 mouse genes, termed Frp2 and Fpr3 (74 and 76%, respectively, at both amino acid and RNA level). Like for the human FPR2/ALX, LXA_4 binds and activates both mice Fpr2 and Fpr3 receptors (Takano, Fiore et al. 1997). We began the project using GFP expression to study the cellular localization of the receptors and to monitor gene promoter activity during on-going inflammation. GFP expression was monitored using fluorescence microscopy (tissue samples) and flow cytometry (cell samples).

In the tissues, as a positive control, immunocytochemistry with a rabbit anti-mouse Fpr2 antibody was also performed on WT samples. Few antibodies in commerce are reactive against mouse Fpr2/3 because most of them react with the human receptor. The FPR2 Antibody (M-73) from Santacruz used here, is a rabbit polyclonal IgG with an epitope corresponding to amino acids 208-280 mapping within an internal region of Fpr2.

In isolated cells like neutrophils and macrophages, GFP expression was analysed by flow cytometry using the FL-1 channel for the natural fluorescence and, in some cases, with a protocol for intracellular staining using an anti-GFP antibody. Quantification of GFP fluorescence revealed that the Fpr2/3 gene is active (or expressed by a higher number of positive cells) in the kidney (~45% fluorescence positivity), followed by the aorta (35%), heart (30%), lung (15%), liver (10%) and spleen (10%). As expected, using the same staining and microscopic conditions, green fluorescence was absent in tissues from WT mice. Immunohistochemistry

staining for GFP in Fpr2/3 KO samples was validated in WT kidney and lung, tissues selected because of their different degree of fluorescence, using an anti-Fpr2 antibody. It was important in my view to demonstrate that the antibody, albeit polyclonal, displayed an at least sufficient degree of specificity since signal was lost in Fpr2/3 KO tissues.

As said above, the kidney expressed highest levels of Fpr2/3/GFP in resting conditions. Interestingly, it has been published that also the FPR2/ALX agonist AnxA1 is expressed in rat kidney: specifically AnxA1 is enriched in epithelia of Bowman's capsule, in the macula densa and in medullary/papillary collecting ducts (Markoff and Gerke 2005). The presence of both Fpr2/3 and AnxA1 in the kidney suggests a probable correlation of expression between the receptor and its agonist (McKanna, Chuncharunee et al. 1992) with downstream implications for some renal functions. Indeed, after an ischemia injury AnxA1 levels and distribution in the kidney are modified with augmented expression, especially in the ascending limb, with approximately a threefold increase after the recovery phase (McKanna, Chuncharunee et al. 1992). Moreover, AnxA1 as a mediator in glucocorticoid-induced cellular response (Kim, Yang et al. 1996, Donnelly and Moss 1998) and due to its is possible involvement as a second messenger of hormone release (Buckingham, Solito et al. 2003), is also important for kidney development and recovery of physiological stress (Markoff and Gerke 2005).

Furthermore, in line with Fpr2/3 gene promoter activity, AnxA1 is also particularly abundant in the lung. Again, this emphasizes the existence of a pattern, with a correlation of tissue Fpr2/3/GFP and AnxA1 expression and potential physiological

functions of this molecule (Wallner, Mattaliano et al. 1986) and its receptor. In the lung, AnxA1 is expressed in alveolar macrophages (Ambrose, Bahns et al. 1992, Kurosu, Takiguchi et al. 2008), alveolar epithelial type II (Ambrose, Bahns et al. 1992) and tracheal gland cells (Jacquot, Dupuit et al. 1990). Cellular and in-vivo studies indicate that AnxA1 is involved in the anti-inflammatory actions of glucocorticoids in the respiratory tract (Hall, Lim et al. 1999). This FPR2/ALX agonist has also a role in the regulation of pulmonary surfactant synthesis and secretion by alveolar epithelial type II cell (Hall, Lim et al. 1999).

Alongside this pattern, in naïve mice AnxA1, like what we have showed for the Fpr2/3 receptors in terms of GFP expression, is almost absent in the liver (de Coupade, Ajuebor et al. 2001). However, after LPS induced inflammation, liver regeneration after partial hepatectomy (de Coupade, Gillet et al. 2000) or tumour (de Coupade, Ajuebor et al. 2001), the levels of AnxA1 in this tissue are increased (de Coupade, Ajuebor et al. 2001). Macrophages-derived AnxA1, as we have recently shown, is also increased and protective during nonalcoholic steatohepatitis (Locatelli, Sutti et al. 2014).

All these examples underline the idea that expression of Fpr2/3 and the protein agonist AnxA1 may be correlated and perhaps tightly controlled. Also they might be regulated by pro-inflammatory stimuli and/or diseases and, then, be part of a circuit involved in the restoration of tissues homeostasis.

Moving to cellular analyses, Fpr2/3/GFP were also expressed in neutrophils, bone marrow cells, bone marrow derived macrophages and peritoneal cells. Specifically,

Fpr2/3 KO blood neutrophils displayed ~5% of GFP+ve events by flow cytometry (raising to 20% after anti-GFP antibody staining). Bone marrow cells and bone marrow derived macrophages showed instead ~10% of GFP positive cells. Resting peritoneal cells exhibited 5% of GFP positive cells, with a much higher degree of positive events (~50%) following anti-GFP antibody staining. Thus, in cell suspension use of an anti-GFP antibody was preferred to achieve significant numbers of positive events, indicating that GFP was quite weak as a stimulus. We note that nowadays enhanced GFP or Yellow Fluorescent Protein are mostly used as reporters since they provide stronger signals (Shaner, Steinbach et al. 2005). Again, also AnxA1 is expressed in all these cells type (Francis, Balazovich et al. 1992, Getting, Flower et al. 1997, Dalli, Jones et al. 2012).

Altogether, monitoring the pattern of expression of GFP as an indication of Fpr2/3 gene activity in resting organs and cells we can conclude that *the Fpr2/3 receptor is expressed in the organs of our interest (heart, lung and kidney) and also in infiltrating cells that characterized the inflammatory response during acute myocardial injury*. This increases our interest in studying the role of the Fpr2/3 receptors in AMI, specifically in the heart and in secondary injury organs.

Furthermore, the data for Fpr2/3/GFP expression and the current literature on AnxA1 expression, suggest a *high degree of co-localization between the receptor and its anti-inflammatory* ligand in tissues like the kidney, the lung and the liver, prompting us to hypothesise that the pair might be operative, or swiftly become operative, after tissues injury or disease.

Although the previous studies from our lab or Ritchie groups have shown that ANX-A1 peptides limit neutrophil-dependent myocardial necrosis (D'Amico *et al*., 2000; La *et al*., 2001; Gavins *et al*., 2005; 2006) and affects the recovery of left ventricle contractive function (Qin, Buxton et al. 2012), no *in vivo* evidence show expression of Fpr2/3 in the heart, its role in AMI and a functional correlation between the whole protein AnxA1 and Fpr2/3 in this tissue. For this reason, Fpr2/3 KO mice and littermate controls (WT) were subjected to the left anterior descending coronary artery model of acute myocardial infarct and studied. It is noteworthy to mention that left ventricle function of sham WT and Fpr2/3 KO mice were analysed by echocardiography and both mice revealed the same normal heart activity.

Animal models of myocardial ischemia reperfusion mimic the clinical scenario where a period of ischemia is followed by the restoration of myocardial blood flow. There are two methods to induce myocardial ischemia in mice: a cryo infarction using a cryo-pen on the surface of the heart, an inaccurate technique with side effects (Atkins, Hueman et al. 1999, Kolk, Meyberg et al. 2009), and the ligation of the left anterior descending coronary artery (LADCA) (Michael, Entman et al. 1995). By occluding the LADCA, the ischemia can be detected immediately as no further blood flow is permitted in the left ventricle, while the surrounding myocardial tissue is almost unaffected. This model is accepted as the model that better shares the clinical and pathological features of AMI. Moreover, this protocol allows comparative and quantitative studies on the pathobiological and pathophysiological aspects occurring in infarction-related myocardial ischemia.

To compare the data published on AnxA1-derived peptide with the main protein AnxA1, the latter was administered to WT mice. Administration before LADCA occlusion afforded significant cardioprotection as compared to vehicle (PBS). However, AnxA1 administration at the beginning of reperfusion afforded a much lower degree of protection emphasizing a role of the protein in the first phase of ischemia/reperfusion injury. Deletion of the Fpr1 gene did not significantly alter the cardioprotective effect of AnxA1, whilst the protein was no longer effective in Fpr2/3 KO mice. AnxA1 through the Fpr2/3 receptors, significantly decreased the levels of Troponin I, a clinical biomarker of cardiac injury, and Caspase 3, a marker of apoptosis and TNFα induced by AMI. These new results provide mechanistic support to the studies conducted over 10 years ago (D'Amico eta l., 2000) and indicate quite convincingly a functional role for Fpr2/3 over Fpr1, making an interesting distinction from the in vitro observations conducted by Ritchie's lab (Ritchie, Sun et al. 2003, Qin, Buxton et al. 2013).

When other FPR2/ALX agonists were tested in WT mice subjected to AMI we discovered that only the AnxA1/FPR2/ALX pair affords a characteristic cardioprotective function outside the one would expect in view of their anti-inflammatory properties. Indeed, though reported to be protective in post-conditiong (Zhao, Shao et al. 2013) and in ischemia-reperfusion injury following cardiac arrest in a rabbit model (Chen, Wu et al. 2013), Lipoxin A_4 (administered at 100 ng i.v. prior ischemia) did not induce a cardioprotection in our AMI setting. The synthetic FPR2/ALX agonist compound 43, administered i.v or i.p prior ischemia, decreased the infarct size induced by AMI but the effect was significantly lower in comparison with the one induced by AnxA1. Also, the effect was not dose

dependent, perhaps indicating the impact of unfavourable pharmacokinetics properties. I also tested the omega-3–derived lipid mediators Resolvin D1 (RvD1, 100 ng prior ischemia) (Serhan, Clish et al. 2000), since the ability of omega-3 polyunsaturated fatty acids to induce protective effects in cardiovascular (De Caterina 2011) and inflammatory diseases (Calder 2003). In our model we could observe that RvD1 decreased infarct size induced by AMI in WT mice, however this cardioprotective activity was not characterised by change in cardiac injury biomarkers like troponin I levels and caspase 3 activity but was only characterised by a decrease in the levels of the neutrophil chemoattractant protein named KC. This was in line with the documented role of resolvin D1 in limiting neutrophil recruitment during inflammation (Norling, Dalli et al. 2012).

Interesting, acute myocardial infarct upregulated mRNA expression of both AnxA1 and Fpr2, and together with the notion that AnxA1 injection increased the induction of Fpr2 mRNA above the level attained by AMI, these data indicate a potential rapid *engagement* of the pathway in AMI. By strict monitoring the levels of CXCL1 and KC together with AnxA1 and Fpr2 mRNA in the heart after 30 min ischemia and 90 min reperfusion we noticed that only Fpr2 mRNA was significantly augmented during ischemia. The increments in CXCL1 and KC mRNA occurred during reperfusion and were associated with detectable neutrophil infiltration. Therefore, there is a specific phenomenon centred on Fpr2 mRNA whereby its induction in the heart, during LADCA occlusion, was not connected with an increase in neutrophils infiltration; I think this would indicate myocardial-specific functions of Fpr2/3 beyond its known actions on immune cells. Indeed, immunofluorescence of Fpr2/3 (GFP) and AnxA1 showed that they were

expressed, and co-localized, in cardiomyocytes. The validity of the assay, hence the importance of the data produced, was confirmed by detection of both AnxA1 and Fpr2/3 in neutrophils (Ly6G positive cells) that had infiltrated the hearts subjected to acute infarct. Finally, differently from Fpr2/3 as discussed above, the AnxA1 mRNA increased selectively during reperfusion, and this could explain why exogenous injection of the protein was active when given at the beginning of ischemia (higher degree of Fpr2/3 availability) instead of during reperfusion (endogenous increase in AnxA1 with potential Fpr2/3 receptor occupation and/or desensitization).

Altogether this large set of data indicates that the *AnxA1/Fpr2/3 circuit becomes operative during the ischemic phase of AMI in the heart*, perhaps to moderate the necessary yet potentially harmful reperfusion phase. In line with this hypothesis striking observations were made when I tested Fpr2/3 KO mice.

During LADCA occlusion a significant proportion (~40%) of Fpr2/3 KO mice perished during the procedure, and those that did survive up to 90 min post-reperfusion exhibited a larger infarct size (+15%, $p<0.05$) than WT animals. The AMI procedure led to necrosis of ~55% of the area at risk in both mice. Acute myocardial infarct also induced a significant increase of Troponin I plasma levels, more marked in Fpr2/3 KO. Hearts of Fpr2/3 KO mice were also characterised by a decrease in AnxA1 protein after AMI, perhaps suggestive of a positive loop whereby Fpr2/3 activation augments agonist generation. A similar in concept, but different in players and assay, has been recently been put forward by Brancaleone et al. (2013) during mesenteric ischaemia-reperfusion.

All together these data indicate that Fpr2/3 play important protective functions in acute myocardial infarct, warranting further analysis of the mechanisms behind these effects. To do this, further analyses were conducted in myocardial and plasma samples of WT and Fpr2/3 KO mice and analysed in comparison with the respective sham mice.

Hearts from Fpr2/3 KO exhibited an alteration of cardiac filament and higher infiltration of neutrophils as seen by IHC and CXCL1 and KC quantification. Fpr2/3 KO myocardial tissue samples contained higher TNFα levels, in comparison with WT mice, an inflammatory marker associated with a worse coronary artery disease prognosis (Sels, Elsenberg et al. 2012). In addition, Fpr2/3 KO mice displayed an unbalance production of pro and anti-inflammatory bioactive lipids as measured by metabolo-lipidomics.

Plasma levels of the pro inflammatory PGE_2, PGI_2, TXB_2 and LTB_4 were higher in the Fpr2/3 KO mice compared to WT mice. On the other hand, the anti inflammatory PGA_1, RvD_2 and the FPR2/ALX agonist LXA_4 could be detected only in WT mice after acute myocardial infarct.

This is reminiscent of data produced in our lab, before the beginning of my studentship, where LXA_4 was not detected in Fpr2/3 KO following mesenteric ischemia suggesting that, collectively, this receptor can be a master-regulator of vascular events that take place during ischemia and reperfusion, with changes in specific readouts that can be associated with each vascular bed or organ.

Moreover, we have recently shown the existence of an endogenous network in anti-inflammation centred on PMN AnxA1 and activated by selective FPR2/ALX agonists like LXA4 (Brancaleone, Dalli et al., 2011). Specifically, addition of LXA_4 to human polymorphonuclear leukocytes provoked a concentration- and time-dependent mobilization of AnxA1 onto the plasma membrane. The FPR2/ALX antagonist blocked the effect. This might be the reason why, after AMI, Fpr2/3 KO mice lacking of LXA4 had less AnxA1 protein in the heart compared with WT mice, a hypothesis discussed above.

Resolvin D_2 is an omega-3 fatty acid-derived mediator with anti inflammatory effects in microbial sepsis (Spite, Norling et al. 2009), obesity-induced adipose inflammation (Clària, Dalli et al. 2012) and colitis (Bento, Claudino et al. 2011). Specifically, RvD_2 is produced physiologically from the sequential oxygenation of DHA by 15- and 5-lipoxygenase through 17-HDoHE (Spite, Norling et al. 2009). Interesting, plasma samples from Frp2/3 KO mice contained higher levels of the resolvin D precursor 17-hydroxy docosahexaenoic Acid (17-HDoHE) but, crucially, lacked RvD_2, which was instead increased by AMI in WT mice. This is another case where, after AMI, Fpr2/3 KO mice are characterised by less pro resolving mediator compared to WT mice: Resolvin D_2 (Resolvins: resolution phase interaction products) is able to decrease neutrophil trafficking to inflammatory loci and promote macrophages influx and phagocytic activity (Spite, Norling et al. 2009).

Aspirin has one of the greatest impacts on acute coronary syndromes affording reduction of AMI mortality. It is beneficial in early therapy, especially immediately on recognition of AMI symptoms, and in long-term use (Antman, Hand et al. 2008).

This is due to its ability to inhibit the formation of thromboxane A2 preventing platelet activation, adhesion and cohesion. However, since RvD_2 was originally identified during resolution in mice treated with aspirin (Serhan, Hong et al. 2002), RvD_2 synthesis and action might be another reason why aspirin is beneficial in these diseases. Moreover, we have recently shown an involvement of Fpr2/3 in the vascular protective properties of aspirin after mesenteric I/R (Brancaleone, Gobbetti et al. 2013). The inability of Fpr2/3 KO to synthetize RvD2 might be at least one of the reasons why aspirin was less effective in this genotype.

All together, this is the first evidence of a hypothetical role of Fpr2/3 in RvD_2 synthesis and aspirin effects in AMI.

In synthesis, so far, data produced with this project established that Fpr2/3 elicits *anti-inflammatory properties* in AMI, that it is expressed in *cardiomyocytes* and that it exerts a specific *cardioprotective activity in the heart* (e.g. data on Troponin I and Caspase 3). How is Fpr2/3 signalling to produce these fundamental actions?

A large number of studies have shown that IL-6 is involved in the induction of the cardioprotective JAK/STAT3 signalling (Negoro, Kunisada et al. 2000). In the heart there is a basal levels of ~40 pg/mg of IL-6 that increases up to 140 pg/mg after one day of myocardial ischemia reperfusion (Danesh, Kaptoge et al. 2008). In the infarcted myocardium the major IL-6R-expressing cells are neutrophils, monocytes and cardiomyocytes (Negoro, Kunisada et al. 2000). Interesting, I could establish a marked increase in IL-6 at 90 min post-reperfusion in the hearts of Fpr2/3 KO mice but this occurred without evident STAT3 activation. These results brought me to

work further on this pathway in the context of Fpr2/3 and AnxA1, using AG490, a janus kinase inhibitor that block the JAK/STAT3 pathway (Seo, Lee et al. 2009, Huang, Yang et al. 2010, Heusch, Musiolik et al. 2011, Duan, Yang et al. 2012, Ottani, Galantucci et al. 2013).

In WT mice, after AMI, AG490 induced Fpr2 mRNA expression yet it prevented the cardio-protective effect of AnxA1. It is clear that when some cardioprotective pathways (like JAK/STAT3) are down regulated, the other protective pathways (like Fpr2/3-AnxA1) are upregulated.

Interesting, the modulation of phosphorylation of STAT3 by AnxA1 has been also recently proposed in tumorgenesis (Li, Cai et al. 2011). Moreover, AG490 also induced TNFα and IL-6 mRNA, pro-inflammatory mediators that, as shown above, were significant higher in Fpr2/3 KO mice after AMI. The lack of STAT3 activity might be one of the reasons why, after AMI, higher levels of TNFα and IL-6 were quantified in Fpr2/3 KO mice in comparison with WT mice. Therefore the cross-talk between the AnxA1/Fpr2/3 and the JAK/STAT3 pathways affords not only cardioprotection but it is also important for the anti-inflammatory activity as measured by cytokines levels.

To summarise, with this set of experiments we have shown for the first time that *AnxA1* and *Fpr2/3* are involved in the induction of the cardioprotective and anti-inflammatory *IL-6/JAK/STAT3* pathway in the injured myocardium.

Ischemia reperfusion injury, after AMI, may also extend beyond the ischaemic area and provoke injury of remote non-ischemic organs resulting in multiple organ

dysfunction syndrome (MODS), which account for up to 30-40% of intensive care unit mortality (Maxwell and Lip 1997, Eltzschig and Collard 2004). Within 24-72h of the ischemic event, the pulmonary system is the most frequently injured organ. Respiratory failure is often followed by hepatic, renal and gastrointestinal dysfunctions (Ware and Matthay 2000).

As we observed by monitoring GFP expression, the receptor Fpr2/3 were also expressed in the lungs and in the kidney. Moreover it is published that in the lungs the receptor, together with their agonists, protects against lung injury (Chen, Le et al. 2010, Barnig, Cernadas et al. 2013, Eickmeier, Seki et al. 2013, Wang, Zheng et al. 2014) and against models of cystic fibrosis (Buchanan, McNally et al. 2013), chronic obstructive pulmonary disease (Bozinovski, Anthony et al. 2013) and pneumosepsis (Sordi, Menezes-de-Lima et al. 2013). In kidneys, the Lipoxin A_4/AnxA1/Fpr2/3 pathway has a potential therapeutic role in renal ischemia/reperfusion injury, acute renal failure and glomerulonephritis (McMahon, Stenson et al. 2000, Leonard, Hannan et al. 2002, Araujo, Truzzi et al. 2010, Facio, Sena et al. 2011). For this reason we have also studied the role of the Fpr2/3 receptors in lungs and kidneys after AMI showing that Fpr2/3 activation also exerted a *protective role in secondary organ injury*.

Specifically, lungs of Fpr2/3 KO mice were characterized by partial destruction of pulmonary architecture and higher tissues infiltration as seen from H&E staining and MPO quantification and higher degree of inflammation (higher IL-6 and less IL-10) compared with WT mice. Kidneys of Fpr2/3 KO mice contained higher KC levels and more TNFα and IL-6 mRNA expression. Interesting, Fpr2 and AnxA1

were upregulated by AMI in both lungs and kidneys. In the lungs they were also colocalized particularly in the epithelial cells of bronchioles underling again the high degree of co-localization between the receptor and its anti-inflammatory protein, hence the likely importance of the AnxA1/Fpr2/3 pathway in AMI-induced secondary injury organs.

In summary, these striking events indicate that, after LADCA occlusion, the AnxA1/Fpr2/3 circuit is protective during the ischemic phase of AMI and in secondary injury organs. The Fpr2/3 KO mice exhibited higher infarct size, myocardial injury and inflammation as compared to WT mice in hearts, lungs and kidney.

Having established the cardioprotective properties of the AnxA1/Fpr2/3 pathway we thought it would be ideal to exploit it to develop a pharmacological treatment able to modulate myocardial reperfusion without the collateral risk showed by other anti-inflammatory remedies already tried and cited. However FPR2/ALX, as stated before, mediates actions elicited by both pro and anti-inflammatory mediators (Chiang, Fierro et al. 2000, Ye, Boulay et al. 2009) and modulates cell responsiveness in a ligand-biased fashion (Li, Cai et al. 2011). The latter point has been chiefly demonstrated in a recent study in macrophages, where both putative 'pro-inflammatory' (or rather activating) FPR2/ALX agonists (such as SAA) and inhibitory agonists (such as AnxA1 and lipoxin A_4) were able to convey their message through this receptor and afford functional antagonism to each other (Li, Cai et al. 2011). In it clear that, to use AnxA1 mimetics as a therapeutically treatment in AMI, AnxA1-biased agonist must be synthetized resulting in an unique

receptor activation that will signal to desirable pathways while sparing those mediating undesirable effects. For this reason we moved to the human FPR2/ALX receptor and we studied the FPR2/ALX binding domains and ensuing signalling required for the activity of the cardioprotective AnxA1 and one of the most pro-inflammatory agonist studied: the acute phase protein Serum Amyloid A (SAA) (Liang, Wang et al. 2000, He, Sang et al. 2003). For completion Compound 43, an anti-inflammatory synthetic compound (Burli, Xu et al. 2006), has also been analysed.

To do so we took advantage of well-characterised stably transfected HEK293 cells with varied FPR1-FPR2/ALX chimeric receptors (Le, Ye et al. 2005). A similar approach but using different chimaeric strategy has been already used to identify the III extracellular loop as a FPR2/ALX binging site for lipoxin A_4 (Chiang, Fierro et al. 2000). An important control useful also for identifying the epitope recognised by commercially available mAbs was the overall amount of receptor expression. It appeared that the anti-FPR2/ALX mAb likely binds to a tri-dimensional epitope formed between the receptor N-terminal domain and the II and III extracellular loops. The antibody revealed similar receptor density on the cell surface as native FPR2/ALX transfected cells. All other chimeras were detected by anti-FPR1 mAb solely. It is evident that the mAbs are able somehow to penetrate within the transmembrane domains, since the anti-FPR2/ALX is active on clone D (containing sequence 40-86 of the native receptor) whilst the anti-FPR1 mAb is not active on clone F (with amino sequence 106-145 of the FPR2/ALX receptor).

Following the binding with its agonists, the FPR2/ALX receptor undergoes a conformational change that allows functional interaction with $G_i\alpha1$, $G_i\alpha2$ and $G_i\alpha3$ and association with $G_{0,}$ G_z and $G_{\alpha16}$ (Migeotte, Communi et al. 2006). This event triggers activation of a variety of signalling pathways including fluxes in intracellular calcium, activation of phospholipase A_2, C and D, phosphoinositide 3-kinase and mitogen-activated protein kinase (Selvatici, Falzarano et al. 2006) and rapid phosphorylation leading to a PLC-mediated receptor desensitization and internalization (Le, Wetzel et al. 2001).

Here, we used as readout for cells activation after FPR2/ALX binding the calcium flux mobilization and the ERK1/2 phosphorylation, since its ability to regulates cell chemokinesis (Wenzel-Seifert, Hurt et al. 1998). In selected experiments modulation of gene expression was also evaluated. We selected three genes from published study (Renshaw, Montero-Melendez et al. 2010): *JAG1* (Jagged1), *SGPP2* (sphingosine-1-phosphate phosphatase type 2), and *JAM3* (junctional adhesion molecule-3) (Renshaw, Montero-Melendez et al. 2010).

Due to the large structure of AnxA1 (Rosengarth, Gerke et al. 2001) and since in presence of calcium it undergoes a conformational change with exposure of its N-terminal domain (Rosengarth and Luecke 2003, Gerke, Creutz et al. 2005), we hypothesise that at least two binding site onto FPR2/ALX for this protein. Hypothesis already proposed quite a few years ago when structural data were scarce (Perretti, Wheller et al. 1995).

Indeed, when the chimeric receptors where tested, the N-terminal domain of FPR2/ALX was sufficient for AnxA1 to elicit rapid downstream responses, whereas

the second extracellular loop was required to provoke more sustained changes, such as those leading to modulation of gene expression. Clone C which could transduce calcium flux and phospho-ERK did modulate JAG1 and JAM3, but clone H with both second and third extracellular loop was nearly as active the native receptor for SGPP2 induction. Analyses conducted with clone C, which contains solely the FPR2/ALX N-terminal region linked to the nearly full FPR1 sequence, corroborated the specificity of full-length AnxA1 for FPR2/ALX, and its inability to engage FPR1. This is in contrast to the ability of short AnxA1-derived peptide that can activate all members of the human FPRs family (Walther, Riehemann et al. 2000, Dalli, Montero-Melendez et al. 2012).

When SAA was tested, we confirmed the genuine agonistic activity of SAA on FPR2/ALX without any activation of FPR1. In terms of receptor domains, analysis of Ca^{2+} fluxes and ERK phosphorylation indicates an interaction with I and II extracellular loop, whilst the N-terminal and III extracellular loop domains are dispensable for the Ca^{2+} response. C43, in line with the ability of small molecules to 'penetrate' inside GPCR loops (Baker and Hill 2007), was active on native FPR2/ALX transfected cells as well as on clones E and F; therefore it binds the I extracellular, III trans-membrane region and II intracellular loop of FPR2/ALX. Even if some work has claimed an involvement of the Fpr1 receptor (mouse counterpart of human FPR1) in C43 anti-inflammatory activity (Sogawa, Shimizugawa et al. 2009, Sogawa, Ohyama et al. 2011), the synthetic compounds did not elicit Ca^{2+} flux responses in FPR1-transfected cells in our setting of experiment. This was in accord with the published data where the in vivo effects of C43 were lost in Fpr2/3 KO mice (Dufton, Hannon et al. 2010).

This section of the project was completed by determining FPR2/ALX homo- and hetero-desensitization, since already reported for AnxA1 and SAA (Li, Cai et al. 2011) and also because of the functional consequences this process may have in modulating cell responsiveness in inflammatory settings (Walther, Riehemann et al. 2000, Dufton, Hannon et al. 2010) and in drug development. AnxA1 engagement of FPR2/ALX II extracellular loop was required for the desensitization of the receptor, as determined by Ca^{2+} readout. The C terminal, in clone H, may also have an important role in FPR2/ALX desensitization, as it has been recently shown (Rabiet, Macari et al. 2011).

In summary, AnxA1 interacts with the N-terminus and/or the II extracellular loop to induce FPR2/ALX receptor activation. SAA interacts with the I and the II extracellular loops of FPR2/ALX. C43 penetrates into the I extracellular loop and the transmembrane regions for full agonism. Of interest here, a recent study proposed a three-point binding model for small molecule ligand interaction with FPR2/ALX (Kirpotina, Khlebnikov et al. 2010) confirming original work conducted with human FPR1 (Lala, Gwinn et al. 1999). These binding site are within the non-conserved amino acid residues 84, 85 (point 1), 163 (point 2) and 284 (point 3). Also in our model AnxA1 binds the FPR2/ALX receptor in the binding pocket that comprises residue 163 (present in clones G). Residues 84, 85 and 163 are in the FPR2/ALX binding region required by C43 and SAA to induce receptor activation.

To conclude, these pharmacological and patho-physiological investigations indicate that the Fpr2/AnxA1 pathway is operative in AMI. On one hand, AnxA1

delivery affords cardioprotection - partly through Fpr2/3 - on the other hand Fpr2/3 is upregulated and protective both in the injured heart and in distant organs, like the lungs and the kindey. We showed for the first time that Fpr2/3 is expressed in the myocardium and that the AnxA1/Fpr2/3 circuit is important in the heart by engaging the IL-6/JAK/STAT3 pathway (Figure 80).

We propose that the AnxA1/Fpr2/3 pathway can be harnessed for the development of novel therapeutics to prevent primary and secondary tissue damage caused by AMI. For this reason we shed initial light on the ligand-specific domains required by AnxA1 for classical FPR2/ALX readouts; in comparison with SAA – which elicits opposite cellular responses.

AnxA1 mimetic that binds the identified FPR2/ALX domains used by the protein to induce a signalling response might be developed avoiding the activation of the receptor by other molecules or pro-inflammatory molecules like SAA. Since the ability of only AnxA1, and not SAA, to activate FPR2/ALX (calcium mobilization, ERK phosphorylation, gene modulation) through the N-terminal domain, AnxA1 mimetics able to bind and activate only the N-terminal might be the perfect molecules to activate the cardioprotective AnxA1/Fpr2 pathway.

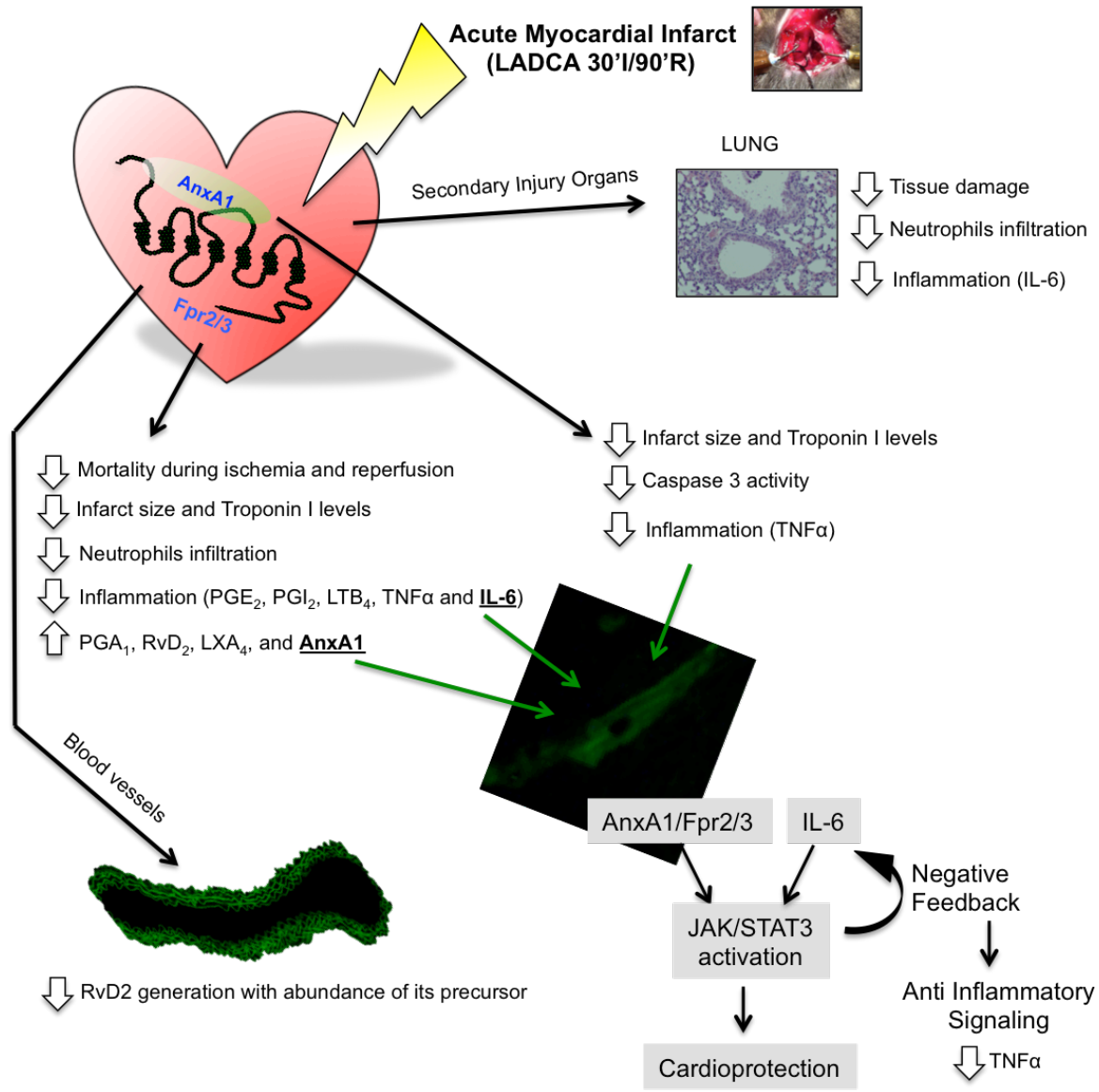

Figure 80: Modulatory properties of AnxA1 and its receptor in controlling local and distant tissue reactivity in AMI.

Pathophysiology and pharmacology indications for a myocardial specific protective circuit centered on the Fpr2/3 receptor and its ligand Annexin A1 with protective effect also in secondary injury organs, like the lungs.

Based on the results produced with this project, future studies will aim to:

- ✓ Investigate the role of the Fpr2/3 receptor in the heart and in cardiomyocyte using the Langendorff heart, an isolated perfused heart assay that removes the effects of Fpr2/3 and AnxA1 given by circulating cells.

- ✓ Investigate the role of the Fpr2/3 receptor in the heart and in cardiomyocyte using tissue-specific conditional knockout. The Fpr2/3 target genes can be specifically inactivated in specific tissues, like the heart and the cardiomyocytes, and functional in all the other tissues and cells.

- ✓ Study the expression of the FPR2/ALX receptor and the AnxA1 protein in human blood samples of acute myocardial infarct patients.

- ✓ Development and analysis of AnxA1 mimetic that binds the identified FPR2/ALX domains used by the protein to induce a signalling response.

I would like to finish this work with some words of my second supervisor Professor Rod Flower. I think they underlie one of the big challenges of the pharmacologists: understand how the drugs interact with living system. We all know the big role that the receptors have in this interaction. A lot of drugs, as well as endogenous molecules, have to bind and activate a receptor to afford their “work”. This

emphasizes the study of the physiological receptors and this is one the reason that led me to choose this project.

"Pharmacology itself is a rather curious discipline. Unlike its sister sciences: physiology, biochemistry, anatomy and so on, it wouldn't have a function at all if we didn't take medicines or drugs. As the cosmologist Martin Rees is fond of noting, we know more about what goes on in the center of a star than we do in the brain of an insect. Whilst we may never completely fathom the complexity of the cell or the physiology of an organism, we are even more at a disadvantage when it comes to understanding drug action. The way in which each new drug interacts with living systems poses a unique challenge to pharmacologists. …There is a good example of this in your bathroom cabinet: aspirin, inhibits the synthesis of prostaglandins and reduces fever, pain and inflammation. Salicylic acid, its chief metabolite and the drug aspirin replaced, does the same – but apparently through a different mechanism entirely." ***Pharmacology 2.0 by Rod Flower, William Harvey Research Institute, 25th Anniversary 2011.***

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